CHEMICAL STUDY OF THE BRAIN IN CASES OF DEMENTIA PRÆCOX.*

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The chemical study of these cases was first suggested to me by Dr. F. W. Mott, F.R.S., on account of the fact that neither microscopic nor macroscopic evidence could in any way account for the symptoms of the disease. That dementia præcox is a clinical entity has, after a good deal of discussion, finally come to be generally accepted. The cases here recorded, especially case 43, which was most carefully studied, are typical and could not be mistaken for any other conditions.

Chemical studies so far made have shown in these cases the existence of a peculiar change in the chemical composition of the brain, consisting of a deficiency in the metabolic or chemical activities of the nervous system, the exact interpretation of which is at present impossible, and probably will remain so until this change is produced experimentally. The foundation for the deficiency here met with lies, probably, in some disturbance occurring during early or embryonic development, as a result of which the brain fails to acquire its full metabolic activity and recuperative power. Such a nervous system can meet simple conditions, but when subjected to the strain of adolescent life it gradually deteriorates, producing the chemical differences observed. This view supplies a chemical or metabolic basis for the idea expressed by Kraepelin, and in a manner supplements the anatomical view elaborated by Bolton;[1] namely, that deterioration of the nervous system cannot take place unless there is an underlying instability of the neuron. This general conception is also not out of harmony with the tendency of the more recent

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[1] Bolton, J. S., *Arch. Neurol.*, 1903, ii, 424; *Jour. Mental Science*, 1905, li, 170, 507, 659; 1906, lii, 1, 221, 427, 711; 1907, liii, 84, 423; 1908, liv, 1, 264, 433.

studies of the psychologists, so well summed up by A. Meyer in the expression, "Mind like every other function can demoralize and undermine itself and its organ and the entire biological economy." [2] It seems inconceivable that such an extreme disturbance of chemical balance as has been observed in the later stages of dementia præcox can ever arise in a nervous system which has a normal amount of recuperative power.

Our chemical observations, which concern largely the distribution of sulphur in different fractions, are given in the following table:

TABLE I.

	Normal.		Dementia Præcox.				
	Case 19.	Case 70.	Case 28.	Case 29.	Case 41.	Case 42.	Case 43.
Protein S	60.2	63.4	64.7	59.7	65.0	66.7	66.6
Lipoid S	27.1	24.0	24.6	28.8	23.0	24.8	23.7
Neutral S	9.5	8.4	5.0	5.6	6.1	3.3	5.9
Inorganic S	3.3	4.2	(5.6)	(5.9)	(5.9)	(5.2)	3.7
Total S in per cent. of dry matter.		0.48					0.48

The methods of analysis have already been described.[3] The significance of the various fractions is as follows:

Protein Sulphur.—Sulphur in protein or proteose combination such as is found in probably all the proteins of the tissues; a part is represented by neurokeratin.

Variation in this fraction: { minimum 1 per cent. / maximum 10 per cent.

Lipoid Sulphur.—Sulphur in combination with lipoids.[4] Found only in the nervous system and there only in the medullated fibres.

Variation in this fraction: { minimum 1 per cent. / maximum 17 per cent.

Variations in protein and lipoid sulphur fractions are within analytical errors.

Neutral Sulphur.—Sulphur in non-colloidal water-soluble combinations, not precipitated by barium chloride direct. This fraction

[2] Meyer, A., *Brit. Med. Jour.*, 1906, ii, 757.
[3] Koch, W., and Upson, F. W., *Jour. Am. Chem. Soc.*, 1909, xxxi, 1355.
[4] Koch, W., *Ztschr. f. physiol. Chem.*, 1910, lxx, 94.

consists probably of a mixture of proteinic acids (resembling those found in the urine) and taurine.

Variation in this fraction: { minimum 27 per cent. / maximum 65 per cent.

The variations in this fraction are the most noticeable, and as they are beyond any possible errors in the method, they form the basis of the conclusion given above.

Inorganic Sulphur.—Sulphates directly precipitated by barium chloride. The estimations in this fraction were subject in the first four pathological cases to a source of error which was eliminated in normal case 70 and dementia præcox case 43. A comparison of these two cases reveals that the variations in this fraction are slight.

The chemical analysis of brains from cases of dementia præcox reveals, then, a variation in the neutral sulphur fraction; in other words, a difference in chemical composition of a nature not so far observed in other forms of insanity, or in cases free from mental disease.

Because of the chemical relationship of this group of sulphur compounds to a similar group in the urine, and in view of the suggestion of Folin[5] that in the urine this group may bear some relation to tissue metabolism, we have reached the tentative conclusion expressed above; namely, that in dementia præcox there exists a condition of metabolic deficiency. We further believe that the absence of demonstrable macroscopic or microscopic anatomical changes in the brain of these cases gives to this chemical finding an especial significance.

Brief protocols of the cases follow.

Case 19.—W. D., Brompton Hospital, London. 1907. Male, age 24 years. Autopsy nineteen hours after death. *Weight of brain.*—1,230 grams. Convolutional pattern good. No wasting. Most of the nerve cells are normal, although a considerable number show chromatolysis, without much destructive change. Some Betz cells show chromatolysis which is generally perinuclear, and resembles that observed in dementia præcox. There is some slight vascular and neurogliar proliferation. *Cause of death.*—Pulmonary tuberculosis. *Duration of disease.*—Eighteen months; in hospital three months. *Diagnosis of mental state.*—Normal. *Previous occupation.*—Printer.

[5] Folin, O., *Am. Jour. Physiol.*, 1905, xiii, 117; Phighini, G., *Arch. Neurol.*, 1909, iv, 220.

Case 70.—J. E. S., Department of Pathology, University of Chicago. May 1, 1908. Male, age 43 years. Autopsy four to five hours after death. *Weight of brain.*—1,400 grams. *Cause of death.*—Diffuse suppurative meningitis. *Duration of illness.*—One week. *Mental condition.*—Normal. *Previous occupation.*—Surgeon.

Case 28.—M. R., Horton Asylum. January 1, 1908. Female, age 17 years. Autopsy five hours after death. Brain sent to Claybury and placed in cold chamber. Forty hours elapsed before material was collected. *Weight of brain.*—1,075 grams. *Cause of death.*—Tubercular salpingitis, tuberculosis of lungs, intestines, and left elbow joint. *Diagnosis of mental state.*—Dementia præcox (katatonic stupor). *Previous occupation.*—General servant. *Length of time in asylum.*—Three months.

Case 29.—C. H., Bexley Asylum. March 2, 1908. Male, age 23 years. Autopsy twenty-six hours after death. *Weight of brain.*—1,165 grams. *Cause of death.*—Lobar pneumonia. No tubercle. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—Laborer. Reached only IVth standard at age of thirteen. *Length of time in asylum.*—Four months and twenty days.

Case 41.—M. D., Horton Asylum. February 19, 1908. Female, age 28 years. Autopsy eighteen hours after death. *Cause of death.*—Tuberculosis of lungs and intestines. *Weight of brain.*—1,095 grams. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—Married, housewife. *Length of time in asylum.*—Three years and nine months.

Case 42.—C. O'C., Horton Asylum. March 3, 1908. Female, age 27 years. Autopsy thirteen hours after death. *Weight of brain.*—1,190 grams. *Cause of death.*—Morbus cordis, fatty degeneration, bronchitis. No tubercle. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—Domestic servant. Single. *Length of time in asylum.*—Four years and eleven months.

Case 43.—F. B., Long Grove Asylum. 1908. Male, age 26 years. Autopsy twenty-five hours after death. Brain sent to Claybury and placed in cold chamber. Forty-seven hours elapsed before material was collected. *Weight of brain.*—1,345 grams. *Cause of death.*—Tuberculous broncho-pneumonia. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—See later history. *Length of time in asylum.*—Nine months.

Details of Case. Family History. Father's father.—Followed the sea, once suspected as a smuggler; died at 65 years. *Father's mother.*—Had two sons by first husband (both alive, 55 and 60 years old). Died of heart failure, age 70 years (?). *Mother's father.*—Age at death not known; lived to good age. No phthisis. *Mother's mother.*—No phthisis. *Father.*—Well-developed, intelligent man, age 53 years. Occupation, butcher. Shows no evidence of alcoholic excess, though not a teetotaler. Mental ability very good. *Mother.*—Rather frail looking, but otherwise in apparently good physical health. Age 55 years. *Brother.*—Age 28 years. Occupation, clerk in a bakery; has been in the South African war. *Sister.*—Age 22 years. Lives at home; well-developed girl in very good physical health. *Father's brother.*—Age 51 years. Occupation, insurance agent. *Father's brother.*—Age 49 years. Occupation, publican. *Father's brother.*—Died in infancy in convulsions. *Mother's brother.*—Age 44 years. Married; has two children, a girl aged 6 years, and a boy 13 years. *Mother's brother.*—Age 40 years. Married; no children. *Mother's brother.*—Died at 40 years.

Cancer of the stomach. *Mother's brother.*—Accidentally drowned at 12 years. *Mother's sister.*—Age 35 years. Married; has three children, a girl aged 16 years, a boy aged 14 years, and a girl aged 8 years. *Mother's sister.*—Died at 42 years of age. Ulcer of the stomach.

No history of tuberculosis or insanity appears.

Patient's History.—Second oldest in the family. Rather quiet as a child, but not surly. Mental development proceeded fairly normally; average progress in school. When eleven years old he received a blow on the head with a brick.

After leaving school he took a position as clerk with a tobacconist. While in that position he had a fall from a bicycle. He was not very sociable as a young man, especially with his sister's friends. At the age of about twenty years he left his situation and the family saw very little of him. During that time he joined the army, was sent to Ireland, deserted twice, and was finally discharged by the army surgeon. At the age of about twenty-two or twenty-three his father took him into his home, but could do very little with him. His father offered him a situation in his shop. Hallucinations began about this time: "There is hidden torture in my inside and under the boards"; "I want reorganizing"; "There are mice all over the place." He was very irregular and peculiar with his food. His mother tells of his having a tomato in his tea. The delusions finally centered about his father, who attempted to be strict with him and whom he finally attacked. One morning he poured something into his mother's tea, which she says made her feel queer and which she got rid of by producing vomiting. At the age of about twenty-three he was sent to the Lambeth infirmary; at the age of twenty-four, to the Fisherton House; and at the age of twenty-five, to Long Grove Asylum where he died at the age of twenty-six. His father denies alcoholic excess or excessive smoking in his son. He was not married and no facts are known about sexual excess.

Admitted to Long Grove Asylum, July 12, 1907. Physical examination. Weight.—126 pounds; Height 5 feet, 9½ inches. General bodily condition fair. *Examination of head.*—Rather poor facial expression. Abundant reddish hair on cranium, average amount on face; freckled complexion. *Examination of neck.*—Thyroid not enlarged. *Examination of nervous system.*—Nothing abnormal. *Examination of vascular system.*—Nothing abnormal. *Examination of chest.*—Flat and sunken beneath clavicles. Note at right apex impaired. Movement and air entry good; no abnormal sounds. *Examination of alimentary system.*—Nothing abnormal. *Examination of gastric system.*—Nothing abnormal. *Examination of urine.*—Not made.

Mental State at Asylum.—January 17, 1908. He sits quietly in his chair, rather huddled up, his expression wooden, and his eyes directed at the floor some distance in front of him. Mucus dribbles from his nose, and saliva occasionally from his mouth, neither of which the patient makes any effort to remove. The hands are blue and congested. If he is addressed, he answers without lifting his head. There is no appreciable perceptual impairment; the patient comprehends immediately what is said to him and his answers are prompt and, in general, more or less relevant. But there is great disaggregation of consciousness; isolated ideas crop up sometimes with the suddenness of an impulse, and between all these isolated elements there is an entire lack of coördination. The patient has no connected idea of his environment or of his

relations in time and space. He gives the year correctly, but suggests that it is April when it is January, that the season is summer and that Christmas occurred ten months ago. Of the self contradictory nature of these statements he has no appreciation whatever. The name of the place he gives as "Fethard." "It is an institution for Protestant clergy." He is not a clergyman himself, but belongs to the "Redemptoristines." Neologisms similar to the last are of frequent occurrence in his conversation. Memory is very defective; isolated reminiscences exist, but their coördination into an ordered whole is impossible. There is no noteworthy difference between his memory of recent and remote events. He is able to read and write fairly well and does simple arithmetic with moderate rapidity, although occasionally with egregious errors. The association of ideas is considerably disturbed. Bizarre associations with no apparent logical continuity are of frequent occurrence, and meaningless phrases are also often observed. When asked what he did at Salisbury, he replies: "A matter of entertainment—mechanical experience"; and in answer to the question, "Have you been in an asylum?" he says, "A matter of bewilderment all around." Although there is no present evidence of the existence of hallucinations or delusions, if he is questioned concerning those mentioned in the certificate (see above), he not only remembers but maintains that the facts were actually as he said: e. g., that "pain came up to him from under the boards," etc. In the present disintegrated state of his mind it is impossible to determine the origin or connection of these various ideas. The affective state is one of marked apathy with complete atrophy of all the higher emotions. Attention is greatly disturbed—he is incapable of any connected train of ideas and any isolated idea irrupting into consciousness is promptly acted upon; for example, he will suddenly get up and say, "I must go now." Although not greatly in evidence at the present time, numerous volitional disturbances have been noted since admission to the asylum, a tendency to preserve attitudes for indefinite lengths of time, resistiveness, impulsive violence, etc. His habits are depraved in numerous ways; he does not employ himself in any way and is extremely untidy.

Progress of Case.—December 4, 1907. Remains in same condition; untidy, defective habits, neglectful of his person.

January 17, 1908. Mental state given above taken at this date by Dr. Hart.

January 23, 1908. Mental state unchanged. He is in very fair health and has made a slight gain in weight (two pounds).

March 24, 1908. Has had a fainting fit; otherwise in his normal condition.

April 13, 1908. Put on sick notice.

April 20, 1908. He has recently been getting thinner and more pallid. During the past fortnight an intermittant temperature has been present, accompanied by evening sweats. Definite signs of phthisis, which appears to be rapidly advancing, are present. Cavitation at right apex, and fine râles and rhonchi over greater part of both lungs. No cough.

April 21, 1908. The breath during the past two days has been intensely offensive and indicates gangrene of lungs. No blood or mucus in stools. Died at 11.10 p. m.

Post Mortem Examination.—April 22, 1908, 12.15 p. m.

Marked tuberculosis of right upper lobe with numerous ragged cavities sur-

rounded by gangrenous areas. *Tuberculous broncho-pneumonia* of left lower lobe; small fibrous area at left apex. Granular kidneys and tuberculous enteritis present. Condition thought to be one of rapidly advancing phthisis developing from old quiescent areas. Right hemisphere taken for chemical analysis.

Histological Examination.—The examination revealed nothing unusual and was not extended, as a study of a large number of the other cases by Dr. Mott has demostrated nothing which can be said to be particularly characteristic of this disease.

In conclusion it gives me pleasure to thank Dr. Mott, F.R.S., and Dr. Wells for material, Dr. Hart for clinical notes, as well as Dr. Bond and the Asylums Committee of the London County Council for the privilege of continuing my studies at Long Grove Asylum.

THE BLOOD SUPPLY OF THE KIDNEY. V. THE INFLUENCE OF THE VAGUS NERVE UPON THE VASCULARITY OF THE LEFT ORGAN.*

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It has been demonstrated by direct measurements of the blood-flow[1] that the kidneys receive a very effective vaso-motor supply by way of the greater splanchnics. The question whether the blood-vessels of these organs are also innervated by the vagus nerve remains, however, still rather unsettled, in spite of the fact that several investigations pertaining to it have been made. Thus Cohnheim and Roy[2] concluded upon the basis of oncometric determinations that the nerves just mentioned are devoid of vaso-motor fibers for this particular circuit. The problem was again dealt with plethysmographically by Bradford[3] and Walravens.[4] These authors also deny the existence of such fibers within the vagal pathway.

Masius,[5] on the other hand, holds that fibers of this type are present, because when chloral or atropin are injected, the stimulation of the peripheral stump of the vagus does not produce an inhibition of the urinary secretion; and, furthermore, vaso-constrictory influences cannot well be excluded, because the inhibition, whenever present, continued for a longer time than the fall in arterial blood-pressure. Arthaud and Butte[6] also observed, during the inhibition of urinary secretion, a decrease in the flow of blood from the opened renal vein. It happened at times that long-con-

* Received for publication, December 7, 1910.

[1] See preceding papers of this series in *Arch. f. d. ges. Physiol.*, 1908, cxxiii, 553; 1908, cxxv, 221; 1909, cxxvii, 143, 148.

[2] *Virchows Arch. f. path. Anat.*, 1883, xcii, 424.

[3] *Jour. Physiol.*, 1889, x, 358.

[4] *Arch. ital. de biol.*, 1896, xxv, 169.

[5] *Bull. Acad. roy. d. sc. de Belg.*, 1888, xv, 528; 1888, xvi. 60.

[6] *Arch. de physiol. norm. et path.*, 1890, series 5, ii, 379.

tinued stimulation of the vagus produced a decrease in the flow of urine, while, at the same time, the blood-pressure increased. Schneider and Spiro[7] observed a cessation of urinary secretion without a reduction of the blood-pressure; and again if the vagus was stimulated slowly and the renal nerves were then torn, a belated increase in urinary secretion appeared, such as might have been incited by a vaso-dilatory impulse.

In view of this diversity of opinion, and because an oncometric tracing cannot be regarded at all times as a true indicator of the vascularity of an organ, we have sought to approach the above problem by determining the renal blood-flow directly by means of the stromuhr. This instrument[8] was connected with the left renal vein of larger dogs, in the manner described in detail in the preceding communication.[9] Chloroform and ether were employed for the narcosis.

On account of its effect upon the general blood pressure, excitation of the cervical portion of the vagi could not be resorted to even after the administration of atropin, since a certain disturbance of the vaso-motor mechanism by this drug seems not improbable. We decided, therefore, to stimulate the vagal fibers below the heart, either directly above the diaphragm and centrally to the gastric plexus, or distally to the plexus between the stomach and the supra-renal capsule.

Having opened the cavity of the thorax in the region of the ensiform cartilage, both vagi were divided and their distal ends placed separately in covered electrodes, which were protected, in addition, by a rubber membrane. In stimulating the nervous pathway between the gastric plexus and the ganglia in the vicinity of the left adrenal body, an exact mode of procedure was impossible on account of the great diversity of the fibers found in this locality. The excitation of the vagi was repeated several times in the course of each determination of the renal blood-flow. Various strengths of a tetanic current were employed.

[7] *Ergebn. d. Physiol.*, 1902, i, Abt. 1, 419.

[8] The recording stromuhr of Burton-Opitz was employed. See *Arch. f. d. ges. Physiol.*, 1908, cxxi, 150.

[9] *Arch. f. d. ges. Physiol.*, 1908, cxxiii, 553.

The records of the stromuhr in all of the five experiments performed by us do not exhibit variations in the flow greater than those occurring normally, and hence we must accept the view that the vagi do not embrace nerve fibers by means of which the vascularity of the kidney may be altered. Negative results were obtained not only on stimulation of the intra-thoracic portions of the vagi, but also on stimulation of the communicating ramus between the gastric and supra-renal plexuses. In the latter case, to be sure, it is not established that this bridge is formed solely by vagal fibers or their postganglionic terminations, because this pathway might also be used by sympathetic fibers in their passage from the ramifications in the neighborhood of the adrenal body to those upon the ventral and dorsal aspects of the stomach. Two facts, however, seem to speak against such a manner of distribution. In the first place, it has been shown by Burton-Opitz[10] that the vaso-motor nerves of the stomach, derived, apparently, solely from the splanchnic system, ascend by way of the plexus gastro-lienalis. To be sure, stimulation of the peripheral ends of the vagi were followed by changes in the vascularity of the stomach as well as of the intestine, but these alterations, which at times bear a striking resemblance to those of vaso-motor origin, were proved to be dependent upon musculo-motor reactions incited with the help of these nerves. Next, we have the present finding, namely, that this bridge appears to be devoid of vaso-motor fibers for the kidney.

Quite different results were obtained with reference to the afferent character of the vagi. When central excitation of the vagi was resorted to at any one of the points indicated above, we obtained, of course, the well-known inhibition of respiratory activity, as well as very conspicuous rises in arterial blood-pressure. Hence the familiar deduction that the vagi represent largely afferent channels, must also be extended to those fibers which pass between the gastric and supra-renal complexes of ganglia. This route, therefore, seems to be set aside for the reception of those impulses from various intestinal organs which are destined to enter central structures by way of the vagi.

Another question presents itself at this time. Is it possible to

[10] *Arch. f. d. ges. Physiol.*, 1910, cxxxv, 205.

produce an active variation in the renal blood-flow reflexly by means of central excitation of the vagi? Both Roy and Bradford have taken oncometric records of the kidney during the stimulation of the central ends of the vagus and sciatic nerves, and both authors agree that the excitations are usually followed by well-marked contractions of the kidney. Bradford characterizes this contraction as sudden and persistent. As the paper in question deals with the innervation of the renal blood-vessels, it may be inferred that the changes in the volume of the kidney serve here as an index for the blood-flow through this organ and are therefore indicative of vasomotor reactions.

With the intention of altering the renal blood-flow reflexly, we have made use of central stimulation of the vagi at the points previously designated and also of central excitation of the cervical portion of this nerve, the nerve on the opposite side being left intact. To test the effects further, we have also performed several experiments in which stimulation of the central end of the divided sciatic nerve was resorted to, the right nerve being the one most frequently tested. In some of the experiments curare was employed in addition to the ether.

In spite of the fact that the stimulations aforesaid have never incited significant reductions in the blood-flow, such as would conform to the well-marked decrease in the volume of the entire organ, described by Bradford, mention must nevertheless be made of certain minor observations. Thus, it seemed remarkable that by far the largest number of stimulations did not produce a change in the volume of flow, even at a time when the very pronounced rises in arterial blood-pressure had fully developed. In the absence of a greater blood-flow through the kidney during the period of increased blood-pressure, we are forced to the conclusion that the renal blood-vessels retain the normal size of the blood-bed tonically. The high blood-pressure does not produce an active or passive enlargement of the blood-bed to accommodate a greater inflow, but causes the blood-vessels to become tonically set, so that the blood-flow remains practically unaltered.

In a previous paper we have proven that profound reductions in the renal blood-flow occur whenever the greater splanchnic nerve is

TABLE I.

Blood-Flow in Left Renal Vein (Experiment IV).

Phase of stromuhr.	Duration of phase in seconds.	Total quantity of blood in c.c.	Quantity of blood per second in c.c.	Blood pressure in mm. Hg.		Procedure.
				Renal vein.	Femoral artery.	
1	11.1	18.6	1.67	12.0	112.6	None.
2	11.4	18.6	1.63	—	—	
3	11.8	18.5	1.56	—	—	Stimulation of central end, right sciatic, 12 cm., 20 seconds.
4	11.4	18.5	1.62	—	118.5	
5	10.6	18.5	1.74	—	131.6	None.
6	11.6	18.5	1.60	—	130.5	
7	10.8	18.6	1.72	—	—	
8	11.4	18.6	1.63	—	116.8	
9	12.0	18.9	1.57	—	—	
10	12.9	19.0	1.53	—	113.2	
11a	4.9	8.6	1.55	—	—	Stimulation of central end, right sciatic, 8 cm., 18 seconds.
11b	9.8	10.9	1.11	10.0	118.0	
12a	8.1	10.0	1.23	—	—	
12b	8.0	10.8	1.35	12.0	140.5	None.
13	13.2	20.2	1.53	—	—	
14	13.1	20.0	1.52	—	131.5	
15	13.0	19.8	1.52	—	—	
16	13.2	19.8	1.50	—	113.0	
17a	4.2	6.4	1.52	—	—	Stimulation of central end, right sciatic, 8 cm., 20 seconds.
17b	9.5	12.6	1.32	12.0–	119.5	
18a	9.0	12.4	1.37	—	—	
18b	4.8	7.2	1.50	12.0	136.5	None.
19	12.9	19.6	1.52	—	—	
20	13.1	19.8	1.51	—	116.0	

stimulated,[11] and that the decrease appears in spite of the high systemic blood-pressure. However, when, the renal plexus is destroyed beforehand, these stimulations cause an increase in the flow in harmony with the high blood-pressure. In a similar manner, we have produced an increased renal blood-flow in the denerved left kidney, on stimulation of the central stump of the right sciatic nerve. Hence, after removal of the central nervous influences, the renal blood-flow becomes subject to general factors and may then be readily altered passively.

Under certain conditions, the stimulations were followed now

[11] *Loc. cit.*

and then by a decrease in the volume of flow, but the reduction never assumed a significant value. At best we have obtained only such quantitative changes as are given in the accompanying record (table I) of a part of experiment IV. In view of the fact that stimulation of direct vaso-constrictory paths, such as the greater splanchnics or the nerves of the renal plexus, readily leads to a cessation of the blood-flow through this organ, some doubt must arise as to the true nature of the reduction observed in these experiments. Without desiring to deny the existence of a reflex vaso-motor mechanism for the kidney, we are nevertheless forced to admit that we have not been able to influence the renal blood-flow reflexly in a measure which would render the vaso-motor origin of these vascular changes unquestionable.

It must be regarded as certain that the changes here noted are not of sufficient magnitude to be recognized as true vaso-motor reactions. This view seems the more plausible in that the reduction appeared altogether too abruptly and showed too great a constancy. If produced actually by vaso-constrictory means, other than mere variations in arterial tonicity, these vascular reactions should have exhibited a slow and gradual onset and should have continued wave-like until some time after the cessation of the stimulation. Hence, as these decreases in the blood-flow do not display the usual characteristics of vaso-constrictory reactions, we are inclined to regard them as being due to a tonic retention of the renal blood-bed.

When the systemic blood-pressure rises in consequence of central stimulation of the vagus or sciatic nerve, the kidney endeavors to preserve a normal blood supply by keeping the caliber of its blood-vessels unchanged. The latter end is accomplished by a tonic setting of its vascular channels, which at times may go somewhat beyond its normal limit and cause such slight reductions in the blood-flow as have been observed in these experiments. It may also be regarded as proved that the kidney plays a very unimportant part in the production of those pronounced rises in arterial pressure which usually appear in the course of the excitations of the central end of the divided sciatic or vagus nerves.

CALCIUM METABOLISM IN A CASE OF INFANTILE TETANY.*

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Quite recently MacCallum and others[1] have studied calcium metabolism in dogs suffering from post-operative tetany in consequence of parathyroidectomy. The administration either of soluble calcium salts or of parathyroid tissue relieves the tetany in such dogs. This work led us to study a case of infantile tetany which was available. The clinical aspects of the case have been reported elsewhere.[2]

The child was a negro girl, fourteen months of age when first seen (December, 1908), at which time the tetany was well marked. No benefit was derived from change of diet or from the administration either of calcium lactate (six grains per day) or of desiccated ox parathyroid (twenty-two grains per day). The child lost weight.

The treatment just described was discontinued on January 4, 1909, and a constant diet (500 cubic centimeters of milk diluted with 500 cubic centimeters of distilled water per day) was begun, which was kept up for two months. During this period the body weight (fourteen pounds) varied but slightly. During the metabolism periods the child was confined to a specially constructed bed, consisting of a Bradford frame. On this frame, in the middle where there was no canvas, a rubber ring pneumatic pad was placed. The buttocks lay in the open space of the pad. The pad rested on a collecting can, in which a wire screen caught the

* Received for publication, December 8, 1910.

[1] MacCallum and Voegtlin, *Jour. Exper. Med.*, 1909, xi, 118; Berkeley and Beebe, *Jour. Med. Research*, 1909, xx, 149; Cooke, *Jour. Exper. Med.*, 1910, xii, 45.

[2] H. J. Gerstenberger, *Cleveland Med. Jour.*, 1909, vii, 671.

feces, the urine being drained off into a bottle. The arms and legs were secured to the bed. On account of constipation, it was possible generally to obtain feces and urine separate from one another. On some days, however, accidental mixing of these prevented estimation of calcium excretion.

The metabolism work began on January 11, one week after the beginning of the constant diet. Rest periods of one to two weeks had to be allowed, to prevent injury to the child from the confinement. The details of our experiments are given in table I, p. 316.

The symptoms of tetany remained well marked during the entire time of the first metabolism experiment (January 11 to February 17), there being no improvement as a result of the administration of calcium lactate (two grains four times a day) or of the subcutaneous injection of emulsion of fresh parathyroid. When the milk diet was discontinued on March 5, the patient improved; and from the latter part of March until about October 1 tetany was entirely absent. We had planned to study the normal calcium metabolism during October, but were prevented by return of the symptoms.

Early in November, while in the stage of partial recovery, the second metabolism experiment was carried out. This furnished complete data for seven days, the urinary calcium being estimated for each day, but the calcium of the milk and of the feces being estimated in aliquot parts of the entire amount for the week. The diet consisted of 1,000 cubic centimeters of milk daily. The symptoms of tetany were in abeyance. This period was, therefore, a latent stage of the disease, and metabolism was presumably more nearly normal than during the first metabolism period. The child was two years old at this time.

The methods of analysis were the same as those used by Renvall,[3] except that the calcium oxalate obtained from the urine was so small in amount that it was estimated by titration with permanganate.

In this table the calcium ingested and excreted is expressed as grams of calcium oxide.

The estimation of calcium of the mixed milk for February 9 to 12 seemed too high, but a second analysis gave the same figure.

[3] Renvall, *Skandinav. Arch. f. Physiol.*, 1904, xvi, 97.

TABLE I.

Date	Calcium ingested.			Calcium excreted.			Remarks.
	In food.	In calcium lactate.	Total.	In urine.	In feces.	Total.	
Jan. 11	0.6135	0.0	0.6135	0.0175	0.4325	0.4500	Age, about 15 months
Jan. 13	0.6300	0.0	0.6300	0.0294	0.6110	0.6404	Jan. 12, excreta lost
Jan. 14	0.7400	0.0943	0.8343	0.0400	0.5630	0.6030	Jan. 14–17, calcium lactate given
Jan. 15	0.6425	0.0943	0.7368	0.0360	0.4555	0.4915	
Jan. 17	0.7400	0.0943	0.8343	0.0498	1.0140	1.0638	Jan. 16, excreta lost
.....							Jan. 17–30, rest period
Jan. 31	0.6000	0.0943	0.6943	0.0255	0.5640	0.5895	Jan. 27–Feb. 2, calcium lactate
Feb. 2	0.7500	0.0943	0.8443	0.0220	0.7150	0.7370	Feb. 1, excreta lost
.....							Feb. 2–8, rest period
Feb. 9	0.9300	0.0	0.9300	0.0165	0.2080	0.2245	
Feb. 10	0.9300	0.0	0.9300	0.0365	1.0740	1.1105	Feb. 11, excreta lost
Feb. 12	0.9300	0.0	0.9300	0.0180	0.8100	0.8280	
.....							Feb. 12–15, rest period
Feb. 16	0.8000	0.0	0.8000	0.0178	[0.7653]		Ox parathyroid subcutaneously
Feb. 17	0.8000	0.0	0.8000	0.0233			Weight, 14 lbs. Tetany present
Nov. 5				0.0185			Age, 2 years
Nov. 6				0.0379			Latent stage of tetany.
Nov. 7				0.0491			
Nov. 8				0.0654			
Nov. 9				0.0960			
Nov. 10				0.0584			
Nov. 11				0.0554			
Total Nov.5–11	10.8100	0.0	10.8100	0.3807	9.0400	9.4207	

The calcium figure for the combined feces, February 16 to 17, can not be considered to represent the full excretion for two days.

Discussion of Results.—First, as regards the excretion of calcium by the urine, the average daily urinary calcium oxide for January 11 to 13 and February 9 to 17 was 0.0227 gram; but for January 14 to February 2, when calcium lactate was given, the daily average was 0.0348 gram. There was, therefore, slight increase of urinary calcium when calcium lactate was ingested.

In the second experimental period in which the conditions were different, namely, the quantity of milk taken, the age and condition of the patient, the urinary excretion was higher, averaging 0.0544 gram per day. For the seven non-calcium days of the first experiment, the percentage of the calcium ingested that appeared as urinary calcium was 2.8, and for the five calcium lactate days it was 4.4; while during the second experiment, the percentage that appeared in the urine was 3.5.

A more important point is the question of retention of calcium. In the case of adults it has been found[4] that there is an approximate balancing of the calcium ingested and excreted, provided the quantity taken in is neither too low nor too high (*i. e.*, between 0.4 and 1.1 gram of calcium oxide per day, the amount differing with different individuals).

In the case of children, there is normally more calcium taken in than is excreted, *i. e.*, there is retention of calcium. On cow's milk the percentage retained is much lower than on breast milk. Blauberg,[5] in the case of a healthy child seven and one-half months old (7,570 grams body weight) on a diet of undiluted cow's milk containing two grams calcium oxide per day, found 44.4 per cent. retention. Cronheim and Müller,[6] with a healthy six months old child (7,800 grams body weight) on raw cow's milk containing 1.5 gram of calcium oxide daily, found 10.6 to 15 per cent. retention. The lower calcium intake in the latter case may explain the lower figure for retention. Cybulski[7] observed 20.8 per cent. retention in a case of tetany in a child seven months old when taking cow's milk. When compared with Blauberg's figures for a child of the same age, this would indicate diminished retention of calcium during tetany. Iddo[8] also reported diminished retention in tetany. In Cybulski's case there was marked increase of retention on breast milk, the percentage of retention increasing as the child recovered, reaching as high as 87.2. In our case the retention during five non-calcium days (January 11 to 13 and February 9 to 12) was 19.4 per cent., and during the calcium lactate days, 11.7 per cent.; while during the seven days of the final experiment it was 12.8 per cent. Calcium administration did not seem to favor calcium retention in the least. Several investigators[9] have found that in the the case of

[4] Bertram, *Ztschr. f. Biol.*, 1878, xiv, 354. See also von Wendt, *Skandinav. Arch. f. Physiol.*, 1905, xvii, 211; Sherman, Mettler and Sinclair, Calcium, Magnesium, and Phosphorus in Food and Nutrition, *U. S. Dept. of Agric., Bull. No. 227*, 1910.

[5] Blauberg, *Ztschr. f. Biol.*, 1900, xl, 19.

[6] Cronheim and Müller, *Biochem. Ztschr.*, 1908, ix, 79.

[7] Cybulski, *Monatschr. f. Kinderheilk.*, 1906, v, 409.

[8] Quoted by Netter, *Compt. rend. Soc. de biol.*, 1907, lxii, 376.

[9] Bertram, *loc. cit.;* Renvall, *loc. cit.;* von Wendt, *loc. cit.;* Herxheimer, *Berl. klin. Wchnschr.*, 1897, xxxiv, 423.

healthy adults retention of calcium occurred when calcium salts were given.

Stoeltzner[10] argued that infantile tetany was due to poisoning of the organism by calcium. This theory would call for increased retention of calcium, which has not been observed. Cerebral excitability has not been found to be associated with increased calcium content of the brain. In the cortex of the brain of children that died of tetany, Quest[11] found less calcium than in the brains of other children of the same age.

Artificial tetany in dogs is, possibly, quite a different disease from human tetany. Calcium salts and parathyroid injection, which are so efficient in removing the symptoms in animals, produced no effect in our case. Furthermore, Cooke[12] found the calcium excretion of normal and of parathyroidectomized dogs to be quite similar, and reports that the calcium content of the brain of tetany dogs is not lower than that of normal dogs.

[10] Stoeltzner, *Jahresb. f. Kinderheilk.*, 1906, lxiii, 661.
[11] Quest, *Jahresb. f. Kinderheilk.*, 1905, lxi, 114.
[12] Cooke, *loc. cit.*

OBSERVATIONS ON THE SUCCUS ENTERICUS.*

By HERMAN O. MOSENTHAL, M.D.

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The following experiments were performed as controls on tests in nephritis for the increased elimination of nitrogen by the intestines. They are reported because they demonstrate facts concerning the origin of fecal nitrogen, which although already known, are not universally appreciated, and because they emphasize, furthermore, the possible importance of intermediate cycles of metabolism.

Animals.—In these experiments, dogs which were kept in metabolism cages and fed on the required diet were used. Only those dogs were retained which took kindly to the cage life and food, and which showed no pathological amounts of albumin in the urine.

Operation.—A resection of a portion (ten to twenty inches) of the lower ileum was made. The severed gut was reunited. The resected gut, with its mesentery and blood and nerve supply intact, was closed by suture at both ends. The distal end was sewed to the abdominal wall. A few days later, after adhesions had formed, an opening was made into the isolated loop, a bronze canula was inserted and held in place by a silver wire purse string suture, forming in this way a Thiry fistula.[1] The fistula was dressed once or twice a day with absorbent cotton and vaseline, the cotton being held in place by a specially devised canvas jacket. To prevent contamination of the abdominal wound with urine, bitches were selected. These operations were performed by Dr. Henry Janeway, of the Research Laboratory of the Department of Surgery, College of Physicians and Surgeons.

As soon as possible the dogs were put on a constant, weighed

*Received for publication, December 30, 1910.

[1] Thiry, *Sitzungsber. d. k. Akad. d. Wissensch., Math.-Wissensch. Kl.*, Vienna, 1864, Abt. iii, l, 77.

TABLE I.

Complete Protocols.

	Date.	Volume of urine in 24 hrs. in c.c.	Weight of dog, in kilos.	Secretion from Thiry fistula.					Nitrogen for 24 hours in gms.						Ammonia N of succus entericus in per cent. of total N of succus entericus.	Total succus entericus for 24 hours in c.c.
				Hours after feeding before collection was begun.	Period of collection, in hours.	Secretion in c.c. for period.	Calculated for 24 hrs.		Intake.	Urine.	Feces.	Succus entericus.		Excess of N of retained succus entericus over fecal N.		
							Secretion in c.c.	Amount N in gms.				Total.	Total minus loss through fistula.			
Dog 1.	1909															
	Dec. 22	435	13.93	Right after	6	86.00	344.0		8.81		.80*					2511
	24				6	90.00	360.0	.50	"		.80	3.68	3.18	+2.38		2628
	28				6	115.00	460.0	.49	"		.80	3.56	3.07	+2.27		3358
	29				6	42.00	168.0	.30	"		.80	2.19	1.89	+1.09		1226
Dog 2.	1909															
	Sept. 21		11.62	Right after	5	11.75	56.4	.30	6.96		.60*	2.57	2.27	+1.67		482
	29				2.75	8.00	65.2	.24	"		.60	2.05	1.81	+1.21	1.55	557
	Oct. 6	230			6	13.00	52.0	.35	"		.60	2.99	2.64	+2.04	3.28	445
	26	285			6	7.50	30.0	.22	"	5.02	.60	1.98	1.76	+1.16		257
	28	225	11.63		6	7.00	28.0	.26	"	5.02	.60	2.22	1.96	+1.36	1.82	239
Dog 3.	1910															
	Feb. 9		7.00	0	6	15.00	60.0	.22	7.35	5.28	.63	1.08	.86	+ .23		300
	10	131		0	6	14.50	58.0	.34	"	5.28	.63	1.69	1.35	+ .72		290
	12	131		23	6	16.25	65.0	.32	"	5.28	.63	1.58	1.26	+ .63		325
	13	131		18.5	3	9.00	72.0	.42	"	5.28	.63	2.09	1.67	+1.03		360
	14	131		20	4	14.00	84.0	.46	"	5.28	.63	2.32	1.86	+1.23		420
				2	3	8.00	64.0	.23	"			1.17	.94	+ .31		320
	15	131		5	2.5	7.00	67.2		"	5.28	.63					336
	16	131		4	3	6.25	50.0	.17	"	5.28	.63	.84	.67	+ .04		250
	17	152		1	5.75	7.00	29.3	.13	"	5.81	.73	.66	.53	− .20		146
	18	152		0	3	3.50	28.0	.13	"	5.81	.73	.63	.50	− .23		140
	19	152		18	3	20.50	164.0	.86	"	5.81	.73	4.29	3.43	+2.70		820
				2	3	3.50	28.0	.14				.68	.54	− .19		140
	21	152		16	3	7.00	56.0	.52	"	5.81	.73	2.61	2.09	+1.36		280
				0	4.5	5.00	26.6	.11				.56	.45	− .28		133
	22	152		22	3	4.50	36.0	.42	"	5.81	.73	2.11	1.69	+ .96		180
				2	3	2.25	18.0	.11				.57	.46	− .16		90
Dog 4.	1910															
	July 30	122	7.13	19	4	5.00	30.0	.30	4.33	3.44	.31	2.47	2.17	+1.86		243
	31	122		21	4	5.00	30.0	.27	"	3.44	.31	2.17	1.90	+1.59		243
	Aug. 1	122		19	4	5.00	30.0	.29	"	3.44	.31	2.39	2.10	+1.79		243
	2	122		20	4	5.00	30.0	.34	"	3.44	.31	2.78	2.44	+2.13		243
	3	122		19	4	8.00	48.0	.56	"	3.44	.31	4.53	3.97	+3.66		389
	4	122		19	4	4.00	24.0	.31	"	3.44	.31	2.53	2.22	+1.91		195
				1	4	10.00	60.0	.13				1.07	.94	+ .63		486
	5	214		19	4	4.00	24.0	.08	"	2.93	.31	.67	.59	+ .28		195
				1	4	8.00	48.0	.22				1.79	1.57	+1.26		389
	6	214		19	4	3.00	18.0	.08	"	2.93	.31	.62	.54	+ .23		146
				1	4	5.00	30.0	.18				1.49	1.31	+1.00		243
	7	214		1	4	10.00	60.0	.23	"	2.93	.31	1.88	1.65	+1.34		486
	8	214		21	4	4.00	24.0	.15	"	2.93	.31	1.18	1.03	+ .72		195
				1	4	3.50	21.0	.10				.82	.72	+ .41		170

*Value estimated, not determined.

diet of hashed lean meat, lard, cracker meal, bone ash, and water. The animals throve, ate heartily, and seemed none the worse for the operation. About six weeks after the insertion of the canula, there were, in one instance, signs of pressure necrosis about the canula, necessitating the discontinuance of the experiment.

In order to collect the succus entericus from the intestinal loop, the dog was suspended from a frame by means of a canvas sling in which were holes for the legs and a hole corresponding with the site of the fistula; a small funnel leading into a graduate was fastened beneath the fistulous opening and the succus entericus was collected for the period. The dogs were kept in this sling for as long as six hours without apparent discomfort, and usually slept during a part of the time.

The nitrogen was determined by the Kjeldahl method, and the ammonia according to Steel and Gies's modification of Folin's method.[2] The nitrogen content and the quantity of the succus entericus secreted by the entire small intestine of the dog for twenty-four hours were estimated by multiplying the amounts obtained during the given period by two factors: (1) by the figure necessary to bring the number of hours up to twenty-four, and (2) by the figure necessary to compensate for the length of the dog's small intestine, as determined at autopsy.

Summarizing the protocols which are given in detail in Table I, on page 320, the following figures are obtained.

TABLE II.

Averages of Observations.

Dog.	Number of observations.	Weight of dog in kilos.	N intake in gms.	N of succus entericus calculated for 24 hours in gms.	N of feces in gms.	N of urine in gms.	The per cent. the N in the succus entericus bears to the N in the			Amount of succus entericus in 24 hours, in c.c.
							Intake.	Feces.	Urine.	
1	3	13.9	8.81	3.14	0.80*	8.00*	35	393	39	2431
2	5	11.7	6.96	2.36	0.60*	5.02	34	393	47	398
3	15	7.0	7.35	1.53	0.68	5.60	21	225	27	282
4	14	7.13	4.33	1.88	0.31	3.18	43	607	59	276

* Value estimated, not determined.

[2] Steel and Gies, *Jour. Biol. Chem.*, 1908, v, 71; Steel, *idem*, 1910, viii, 365.

At the first glance the remarkable fact becomes apparent that the nitrogen secreted by the small intestines exceeds considerably that excreted in the feces. It is probable, however, that the nitrogen content of the succus entericus as given here is too small rather than too large, for the following reasons:

1. The secretion collected from a Thiry fistula does not represent the full quantity poured forth by the intestinal mucous membrane, since some of the material is reabsorbed before collection.[3] This is well shown in dog 1. In this animal the opening of the fistula was so wide that it was impossible to keep the canula in position, and after a week's observation prolapse of the loop occurred through the large stoma. This wide opening, however, evidently permitted a freer flow of juice with less reabsorption, and accounts for the greater amount of fluid as compared with those from the other cases.

2. After a lapse of time the Thiry fistula secretes less actively than at first.[4] This is apparent in the complete protocols, where the later periods show diminished secretion. The table of averages includes the late as well as the earlier observations.

3. The amount of succus entericus secreted in the upper portions of the small intestine is greater than that produced in the regions approximating the ileocecal valve.[5] In the present series of operations, the loops were taken from the lower ileum and may be considered to have yielded the minimal quantity of succus entericus obtainable from an equivalent section of the small intestine. Thus the excess of the nitrogen secreted by the small intestine over that excreted in the feces for any given period is very probably even greater than is indicated in the charts.

It has been known for some time that the gastro-intestinal tract secretes nitrogen, which may make up the whole or a great part of the nitrogen contained in the feces. Hermann[6] and F. Voit[7] paved the way for the recognition of this fact by very suggestive experi-

[3] Frouin, *Compt. rend. Soc. de biol.*, 1905, lviii, 653. O. Cohnheim, Nagel's Handbuch der Physiologie des Menschen, Braunschweig, 1907, ii, 593.

[4] Frouin, *Compt. rend. Soc. de biol.*, 1904, lvi, 461.

[5] Frouin, *Compt. rend. Soc. de biol.*, 1904, lvi, 461.

[6] Hermann, *Arch. f. d. ges. Physiol.*, 1890, xlvi, 93.

[7] F. Voit, *Ztschr. f. Biol.*, 1892, xxix, 325.

ments. Both of these observers worked with dogs in which loops of small intestine had been isolated and sewed end to end to form a ring (Hermann), or closed at either extremity (Voit), and subsequently dropped back into the peritoneal cavity. At the end of several weeks such loops contained a feces-like material, identical in appearance with the feces of starvation and those of a meat diet. Voit on further investigation found that, per square meter of intestinal surface, this retained secretion, on the one hand, and the feces, on the other, had a like weight and nitrogen content. This indicated that there was no food residue in the intestine after a meat diet; that the salivary, gastric, biliary, and pancreatic secretions were completely reabsorbed; and that the feces were derived, under these dietary conditions, solely from the secretion of the small intestine. These deductions have been corroborated by numerous experiments. They are also borne out by the observations presented in this paper. The succus entericus contains waste products, which, on a readily absorbable diet furnish the nitrogen of the feces, equal in amount to about 10 per cent. of the ingested nitrogen. The succus entericus may contain other substances as well, since Voit has shown that the bulk of the feces may very readily be accounted for by the succus entericus.

On glancing at the figures in table II it is seen that the nitrogen secreted by the small intestine varies between 225 and 607 per cent. of that found in the feces. The greater portion of it is, therefore, reabsorbed. The succus entericus contains nitrogen to the amount of 21 to 43 per cent. of the nitrogen intake. Averaging these figures (see table II), it is found that the nitrogen contained in the juice of the small intestine is equal to 34 per cent. of the total nitrogen intake; deducting the 10 per cent. lost in the feces (somewhat less in these cases), it is clear that the remaining 24 per cent. is reabsorbed. Adding to this amount the nitrogen of the bile, pancreatic juice, gastric juice, and saliva, it is evident that nitrogen equal to more than one quarter of the food nitrogen passes into the physiological exterior of the body—the alimentary canal—to be subsequently reabsorbed. In view of this fact, it seems probable that the intestine has, in addition to its excretory function, resulting in the production of the feces, and its digestive function, resulting

in the absorption of the food, a third function, *i. e.*, that of forming a link in the "circulation" of various products, such as the nitrogen or the bile pigments.[8] That this reabsorption is of great metabolic importance is indicated by the amount of nitrogen involved, this being the equivalent, on a mixed diet, of 25 per cent. of the nitrogen intake.

Previous observations and experiments have shown that a part at least of the fecal nitrogen is an excretion, since nitrogen occurs in the feces of starvation. In three instances of experimental starvation in man, the fecal nitrogen amounted to 0.32, 0.11, and 0.2 gram.[9]

In dogs there is a similar excretion in starvation.[10] The passage of food through the alimentary tract increases the amount of fecal nitrogen, regardless of the nitrogen content of the food. This is another indication of the excretory power of the intestines, which is readily appreciated when the amount of nitrogen in the feces on a nitrogen-free diet is compared with that in starvation. Thus in man, on a nitrogen-free diet, 0.54, 0.87, and 0.78 gram of nitrogen[11] were found daily in the feces, these amounts showing a considerable increase over the starvation figures quoted above. In dogs similar conditions have been observed. A 17.5 kilogram dog, whose feces during starvation contained daily 0.14 gram of nitrogen, produced 0.24 and 0.57 gram when taking 132 and 305 grams of nitrogen-free food.[12]

Thus it is seen that the nitrogen in the feces is, to a very great extent, a secretion of the alimentary tract. On the other hand, this is not true under all conditions. The notable exception is found in cases of vegetarian diet which results in a large amount of unabsorbed food residue. On a mixed vegetarian diet, nitrogen equal to 34 per cent. of the nitrogen intake was found in the feces;[13] in a similar case, during two weeks' observation, 42 per cent. was

[8] Howell, A Textbook of Physiology, Philadelphia, 1909, p. 785.

[9] F. Müller, *Arch. f. path. Anat.*, 1893, cxxxi, Suppl., 18, 67; *Berl. klin. Wchnschr.*, 1887, xxiv, 433.

[10] F. Müller, *Ztschr. f. Biol.*, 1884, xx, 327.

[11] Tsuboi, *Ztschr. f. Biol.*, 1897, xxxv, 68.

[12] Rieder, *Ztschr. f. Biol.*, 1884, xx, 378.

[13] Rumpf and Schumm, *Ztschr. f. Biol.*, 1900, xxxix, 153.

found;[14] and on a diet of rye bread, 40 per cent.[15] Many other results might be cited, showing how some forms of food leave large fecal residues and how their remnants may be readily identified in the feces. On an average diet, however, very little of the fecal mass can be attributed directly to the food. Thus the feces from an individual on a meat or sugar diet have approximately the same appearance as those of starvation, both being a pitchy black mass in which microscopical examination shows no food residues.

The fecal nitrogen does not increase in direct proportion to the amount of nitrogen ingested in the meat. Thus in dogs, after successive feedings of 500, 1,000, 1,500, 2,000, 2,500 grams of meat, the fecal nitrogen instead of increasing in the proportions 1 : 2 : 3 : 4 : 5, as we should expect if there were an unabsorbed residue, shows an increase according to the proportions 1 : 1.8 : 2.0 : 2.2 : 3.0.[16]

Another example of the independence of fecal nitrogen, on a readily absorbable diet, is seen in the case of a nursing infant which in two different three day periods received respectively 8.06 and 3.30 grams of nitrogen in its milk. The fecal nitrogen for the former period was 0.62 gram, for the latter 0.61 gram.[17] On two diets containing respectively 0.31 and 0.22 grams of nitrogen, an individual excreted 1.52 and 1.50 grams of fecal nitrogen.[18] Many similar illustrations of the importance of intestinal secretion as a factor in the origin of the feces might be cited. Wherever careful work has been done, the same facts are indicated.

Thus the evidence of the experiments here reported toward a proof of nitrogenous secretion on the part of the small intestine is seen to be wholly in accord with the results of earlier experiments. F. Voit, in the report referred to above, assumed that a reabsorption of bile and pancreatic juice occurs. These two secretions contain a considerable amount of nitrogen. In dogs the biliary nitrogen amounts to 0.5 to 2.5 per cent.[19] of the urinary nitrogen; according to another observer, it equals 6.0 to 6.5 per

[14] C. Voit, *Ztschr. f. Biol.*, 1889, xxv, 232.
[15] Rubner, *Ztschr. f. Biol.*, 1901, xlii, 261.
[16] F. Müller, *Ztschr. f. Biol.*, 1884, xx, 327.
[17] Orgler, *Monatschr. f. Kinderheilk.*, 1908, vii, 135.
[18] Renvall, *Skandinav. Arch. f. Physiol.*, 1904, xvi, 94.
[19] C. Voit, *Ztschr. f. Biol.*, 1894, xxx, 523.

cent.[20] In man the pancreatic juice contains as much as 0.5 gram of nitrogen per day.[21] If these materials are reabsorbed, as Voit claims, we may likewise assume that a reabsorption of the nitrogen of the succus entericus takes place.

It is not intended to give the impression that the nitrogen found in the succus entericus on a mixed diet is constantly 35 per cent. of the nitrogen intake. The methods here employed of collecting the intestinal secretions and making calculations therefrom for twenty-four hour periods for the whole animal, are open to some obvious objections. They are, however, the best available at the present time, and probably yield results which at least approximate the truth. Therefore, emphasis is laid on the fact that considerable nitrogen is reabsorbed from the intestine, and the percentage figures, in spite of their limitations, give some conception of the amounts of nitrogen involved.

The quantities of the succus entericus, as calculated for these animals, are considerable, being comparable to the volume of the urine. The figures given above for the estimated amounts are probably too small rather than too large. The volume of the succus entericus obtained from dog 1 probably gives a truer indication of the average total quantity than that taken from the other animals. In this instance the succus entericus, calculated for twenty-four hours, was equivalent to about one-fifth of the animal's body weight. That all of the secretions of the gastro-intestinal tract are very copious is a well-known physiological fact. It has been claimed that the combined secretions for twenty-four hours are more than equal in volume to the individual's blood serum,[22] thus pointing to a general extensive reabsorption.

The amount of secretion from the small intestine was found to some extent to be dependent upon the food intake. In dog 3 the volume was distinctly less shortly after a meal than when the collections were made some hours later. At times dog 4 showed the same condition as dog 3; in other instances, however, these differ-

[20] Spiro, *Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1880, Suppl., 50.

[21] Glässner, *Ztschr. f. physiol. Chem.*, 1903, xl, 465; Schumm, *idem*, 1902, xxxvi, 292.

[22] O. Cohnheim, Nagel's Handbuch der Physiologie des Menschen, Braunschweig, 1907, ii, 557.

ences in quantity were reversed, and the periods immediately following food intake showed a larger amount of intestinal juice than those observed several hours later. The experiments point, in general, to a more active secretion on the part of the small intestine at the time that it might be expected to contain food, but the results are not sufficiently uniform to warrant insistence on this point.

In some experiments previously reported, it was claimed that the succus entericus was absent at certain times. In the present series of experiments this phenomenon was never observed. Since the distal portion of the intestinal loop was sewed into the abdominal wall, normal peristalsis was sufficient to expel the contained secretions, especially as the fistula was constantly kept patulous by the inserted canula. A neglect of these two factors might result in reabsorption of succus entericus and thus lead to the assumption that no secretion had taken place during a given period.

Only one attempt was made to obtain an analysis of the succus entericus. In three observations on dog 2 the percentage of ammonia nitrogen of the total nitrogen of the succus entericus varied from 1.55 to 3.28.

SUMMARY.

Nitrogen equivalent to about 35 per cent. of the nitrogen intake, though not necessarily derived directly from it, is daily secreted in the succus entericus in dogs on a mixed diet; about 10 per cent. is excreted in the feces, and about 25 per cent. is reabsorbed. The amount of reabsorbed nitrogen-containing material is considerably larger if the bile and pancreatic secretion are included.

The metabolic significance of this reabsorption can only be surmised. That it is probably of great importance is indicated by the fact that in the experiments here recorded the amount of nitrogen reabsorbed equaled approximately 25 per cent. of the nitrogen intake.

ON ABSORPTION FROM INTRAMUSCULAR TISSUE.*

By J. AUER and S. J. MELTZER.

(From the Department of Physiology and Pharmacology of the Rockefeller Institute for Medical Research, New York.)

About five years ago we[1] published the results of a series of experiments upon a comparison of the rate of absorption of solutions from within the muscles and the subcutaneous tissue. We tested the rate of absorption with substances which give a definite reaction; for instance, the effect of adrenalin upon blood-pressure and upon the pupil in animals in which the corresponding cervical ganglion was removed, the effect of curare, and of fluorescein.

The effect of adrenalin upon blood-pressure was studied especially on account of the objective graphic evidence which it furnishes. The investigation brought out the definite result that the absorption after intramuscular injections is far superior to that after injections into subcutaneous tissue, approaching in its effectiveness that of an intravenous injection. Our experiments were made on rabbits and the results obtained were constant. We discussed in our paper the possibility of the intramuscular injection being in reality an intravenous one and brought forward a number of facts which seemed to us to be sufficient to refute such an assumption. Since the appearance of our communication statements have been published on the subject of intramuscular absorption of adrenalin in which the authors arrived at conclusions different from ours. Patta[2] states that in his experiments neither the intramuscular nor the subcutaneous injection of adrenalin ever produced a rise of blood-pressure, and that in his opinion our favorable effects with intramuscular injections resulted from the fact that our hypodermic needle was inserted accidentally into a vein. In a communication

* Received for publication, January 6, 1910.

[1] Meltzer and Auer, *Jour. Exper. Med.*, 1905, vii, 1.

[2] Patta, *Arch. ital. de biol.*, 1906, xlvi, 463.

before the New York Academy of Medicine, Wallace[3] stated that in his experiments an intramuscular injection of adrenalin in rabbits acts indeed practically like an intravenous injection, but he was of the opinion, "that the injection was in truth an intravenous one, that the manipulation resulted in the tearing of a small vein and that some of the adrenalin gained entrance into a vein."

We have recently gone over the subject again and carried out a series of experiments under conditions which, in the first place, exclude the possibility of the needle having entered the lumen of a muscle vein. Our results furnish at the same time a possible explanation for the surprising failure of Patta to obtain an evident effect of adrenalin upon blood-pressure by intramuscular injection. As in the previous investigations, we experimented chiefly upon rabbits, using the rise of blood-pressure after injection of adrenalin as an indicator for the absorption. The injections were made, as in the previous experiments, into the lumbar muscles.

At the outset we may say in general that in employing these lumbar muscles there is no difficulty whatsoever in demonstrating the superiority of the absorption from the muscles over that from the subcutaneous tissue. This was easily accomplished by the simple method of injecting adrenalin with a hypodermic needle. It practically never failed to bring out an immediate definite rise of blood-pressure when the injections were made into the lumbar muscles, while after a subcutaneous injection the effect is inconstant and insignificant. We may mention here that in the present investigation this difference came out in a striking manner also in using potassium cyanide. A minimum dose was found which killed rabbits in a short time by an intramuscular administration, while by subcutaneous injection, the same dose was not fatal, and sometimes not even manifestly toxic. We may refer here also to the experiments of Joseph and Meltzer[4] with magnesium salts, in which the difference between the intramuscular and the subcutaneous injection was very evident.

In order to exclude the possibility of the needle being in a muscle vein, a number of experiments were carried out in the following

[3] Wallace, *Med. Rec.*, 1907, lxxi, 876.

[4] Joseph and Meltzer, *Jour. Pharm. and Exper. Therapeutics*, 1909, i, 373.

manner: we exposed in rabbits the sacro-lumbar muscles and made a small slit in them, through which a large glass cannula was bored slowly and gradually into the muscle tissue where it was safely secured by means of needle and thread. The cannula was usually one of the largest which we employ in the laboratory for the jugular veins of dogs, that is of about five millimeters diameter; it surely

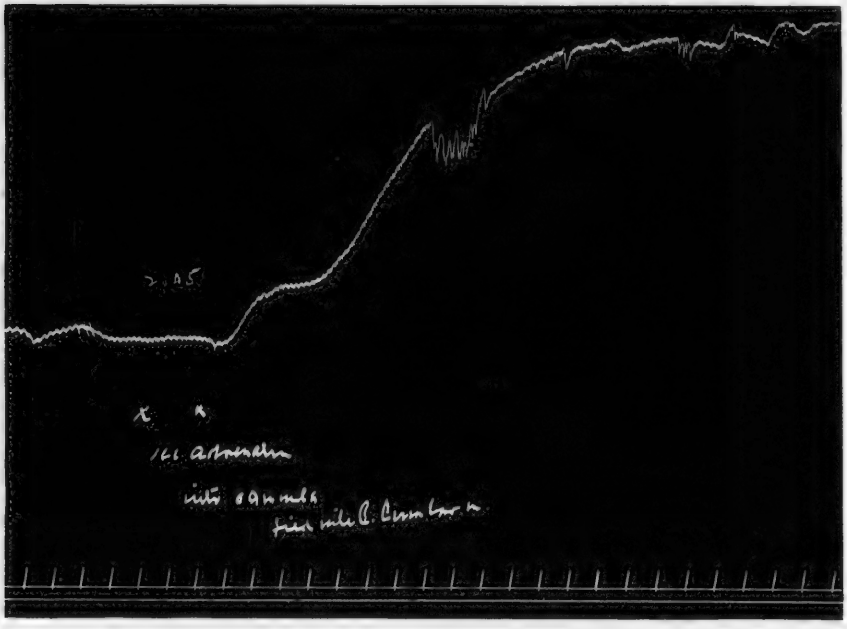

FIG. 1*a*. During the interval marked by crosses on the tracing, 1 c.c. of adrenalin was injected into a cannula which had been tied into the lumbar muscles of a rabbit. The injection was given at 2:05 p. m. Note prompt and considerable rise of blood-pressure.

All the tracings represent the carotid blood-pressure in rabbits taken by means of a mercury manometer. The base line is the straight one beneath the time marking. Time is recorded in four second intervals in all tracings.

could not have entered into the lumen of a muscular vein. A feather was introduced to the bottom of the cannula, to test for the presence of blood; in most instances not even a stain of blood was brought back. The cannula was kept open and free for some-

time, to admit air and hasten the clotting and drying of the blood and thus close the openings in the veins, if any of them were torn; in one case the cannula remained open for forty-five minutes. Then the cannula and the piece of rubber tubing, which was attached to it, were filled with adrenalin and the tubing clamped, after which we again waited a few minutes. The presence of the adrenalin at the bottom of the cannula could assist further, if necessary, in clotting the blood and closing the torn vessels. The simple presence of adrenalin caused no rise of blood-pressure. The carotid and blood-pressure arrangement were prepared in the usual manner. Now one cubic centimeter of adrenalin was injected through the rubber tubing into the lumen of the cannula; in these injections the point of the needle remained in the lumen of the cannula and did not come in contact with the muscle tissue. In experiments so arranged, the cannula could not have been in the lumen of a vein, and if any of the veins were torn by the insertion of the cannula, the openings were closed, at all events at the time when the injection took place. Now as to the results. Practically without exception, soon after the injection was finished, an unmistakable rise of blood-pressure set in, which, in many respects, resembled the rise caused by an intravenous injection of adrenalin (see Fig. 1*a*). Furthermore, even at the repetition of an injection after half an hour, a similar positive result was obtained, although in his case the effect was somewhat less strong than the one produced by a first injection (see Fig. 3).

FIG. 1*b*. Same rabbit. Tracing taken twenty-eight minutes after the injection recorded in Fig. 1*a*. The blood-pressure was still higher than it was normally.

In these experiments the observation was further made that the rise of blood-pressure brought on by intramuscular injections of adrenalin, executed in the manner just described, lasted a good deal

longer than the usual duration of the rise of pressure following an intravenous injection. We have already called attention to this feature in our first paper.[5] In one case of our present series of experiments more than half an hour passed before the pressure returned to its original level (see Figs. 1*a* and *b*).

It is therefore evident that the rapid absorption of adrenalin from the intramuscular tissue in these experiments did not come about from the needle being in a vein or from the entrance of adrenalin

FIG. 2. Same rabbit. During the interval marked by crosses, 1 c.c. of adrenalin was injected into a cannula which had been ligated into the exposed gluteal muscles. No rise of blood-pressure occurred.

into the openings of veins which became torn by the insertion of the needle. Our theory is that adrenalin enters the circulation through the walls of the blood capillaries which are abundantly present in the muscle tissue. The entrance is enforced by the increased pressure which the injection produces within a muscle firmly enveloped in a resistant fascia. Only a small fraction of the quantity of the injected solution present in the muscle tissue enters the circulation at a time, but in such small quantities it continues to penetrate the capillary wall as long as the solution is present in the muscle in a sufficient quantity to maintain the necessary degree

[5] *Loc. cit.*

FIG. 3. Same rabbit. One cubic centimeter of adrenalin was injected again into the cannula tied into the lumbar muscles. A good rise of pressure occurred, in spite of the fact that 1 c.c. of adrenalin had been injected into the same place before.

of additional intramuscular pressure. We would state now that in our opinion the massive structure of the muscle and the resistance of the fascia surrounding the mass of muscle tissue are important

factors in maintaining the increased pressure and thus favoring the rapid absorption from the muscle tissue. Whether the injected solution passes through the normal stomata of the vascular walls or through abnormal openings artificially produced by the force of the injection, is a question which would be difficult to decide experimentally, and it is not important enough to spend time in a laborious attempt at its solution. We may say, however, that, considering the ever-readiness of the muscle tissue for activity, it is only reasonable to assume that the walls of its capillaries are

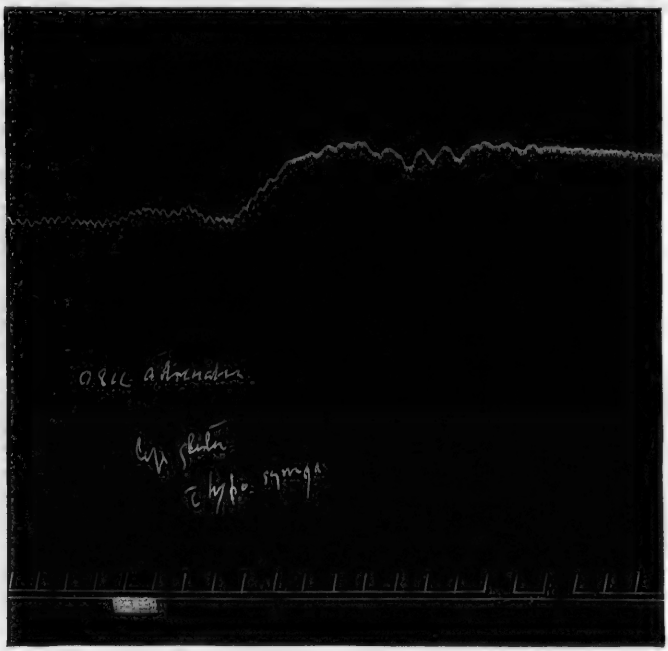

FIG. 4. Another rabbit. In this experiment 0.8 c.c. adrenalin was injected by the ordinary method (hypodermic needle) into the gluteal muscles. The record shows a moderate rise of blood-pressure.

especially prepared for a rapid and easy exchange of metabolic products.

In our previous paper we stated that in injecting into muscles

other than those of the lumbar region, "care has to be taken that the needle remains within the muscle, as with the thin muscles of the animal, it might easily happen that the needle enters the loose areolar tissue between layers of muscles, in which case the effect will be the same as from the subcutaneous tissue." In view of the fact that just at present the gluteal muscles are frequently used in practical therapeutics for the purpose of giving "deep injections," we carried out a series of experiments in which the injections into the lumbar region were compared with those given into the gluteal muscles. The injections of adrenalin were given either simply through a hypodermic needle or through a glass cannula carefully tied in the muscles in the manner described above. The comparisons were made on the same animal, the adrenalin being administered first into one group of muscles and later into the other group. Since in our experience the effect of the first injection of adrenalin was always better than that of subsequent injections, the order of the injections was changed in different animals, giving, for instance, in one animal the first injection into the lumbar, and the second into the gluteal muscles, and reversing the order for the next animal. The results were unmistakable and remained the same no matter what the order of the injection was. While the injections into the lumbar muscles, as stated before, practically never failed to bring out promptly a characteristic reaction, the effects produced by intragluteal injections were, to say the least, unreliable. Either there was practically no effect at all, or the effect was insignificant compared with that obtained from the lumbar muscles (see Figs. 2 and 4 and compare with Figs. 1*a* and 5).

The striking difference in the rate of absorption between the two groups of muscles has its origin, we assume, in some anatomical differences. The mass of the lumbar muscles is nearly round, massive, and of a dense texture, and is tightly encased in strong, nearly inelastic fascia. The mass of the gluteal muscles, on the other hand, is arranged in flat layers, consisting of coarse, loosely connected fasciculi. It seems further that the gluteal muscles are only loosely and incompletely covered by their fasciae. The relation of the fasciae to the rate of absorption from the muscles they envelope, may be important for two reasons: first, it may assist in main-

taining a higher intramuscular pressure and thus favor a more effective absorption, and secondly, it prevents the escape of the injected solution into the adjacent loose connective tissue. When a solution is injected into the mass of the gluteal muscles, it often fails to be rapidly absorbed because, perhaps, on the one hand, the

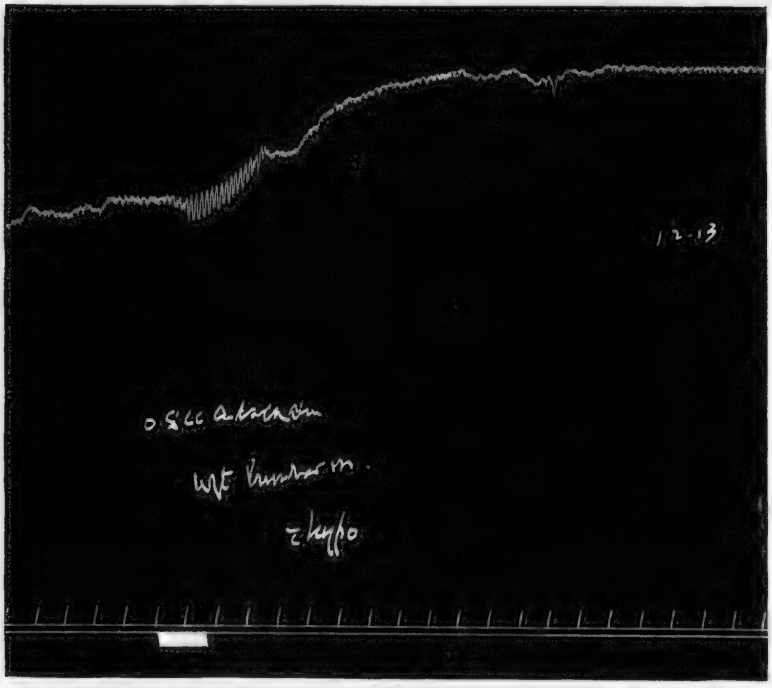

FIG. 5. Same rabbit as in Fig. 4. Eight-hundredths cubic centimeter adrenalin was injected by hypodermic needle into the lumbar muscles of the left side twelve minutes after the injection into the gluteal muscles. The record shows a good and prompt rise of blood-pressure.

injection fails to cause there an effective rise of an intramuscular pressure and, on the other, the solutions, perhaps, frequently escape into the loose connective tissue abundantly present in some parts of the muscles and their neighborhood.

We may record here also the following experiments, based upon the anaphylactic reaction in guinea pigs. In highly sensitized pigs

one cubic centimeter of horse serum, injected into the lumbar muscles either through a glass cannula, as described above, or directly by a hypodermic needle, kills the animal in the same acute manner as an intravenous injection. Such a prompt and rapid result can not be obtained by a subcutaneous injection, nor was it forthcoming when the injection was given into the gluteal muscles. However, the dose necessary for a prompt effect by an injection into the lumbar muscles is at least twenty times as large as the one which would cause an acute anaphylactic death by an intravenous injection.[6]

We may mention here briefly that in dogs the favorable effect from injections into the lumbar muscles were obtained only from large animals and only when the muscles were exposed and we made sure that the needle was within the muscle tissue. In these animals the lumbar muscles do not present a large mass.

The results which we obtained in this investigation may perhaps help us to understand the cause of the failure of Patta to obtain a rise of blood-pressure by intramuscular injections of adrenalin. Patta probably did not inject into the lumbar muscles, and in using other muscles he might not have taken special care to inject exclusively intramuscularly, at least he does not mention it especially. The adrenalin which he believed to have injected intramuscularly might have found its way into the connective tissue between the layers of muscles. In fact, in searching for the presence of adrenalin within the muscle, Patta states expressly that in cutting out the muscles into which he believes he had injected it, he took along also some of the adjacent tissue.

CONCLUSIONS.

Injections of adrenalin or other substances into the lumbar muscles of rabbits are followed by a rapid absorption in whole or in part of these substances.

The rapid absorption is not due to the needle being within a vein or to the tearing of veins by the needle during its insertion.

Similar injections into gluteal muscles fail to give reliable results.

[6] Gay, Southard and Fitzgerald (*Jour. Med. Research*, 1909, xxi, 21) who injected horse serum into the lumbar muscles of guinea pigs (see experiment 2, p. 23) believe that the favorable result was obtained because the injection was a "paraneuraxial" one.

THE RELATIONS OF THE INTERSTITIAL CELLS OF LEYDIG TO THE PRODUCTION OF AN INTERNAL SECRETION BY THE MAMMALIAN TESTIS.*

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Plates XXX–XXXVI.

The questions considered in this paper are, "Does the mammalian testicle, in addition to the formation of sperm, furnish an internal secretion essential to the normal development of the organism?" and, "If so, which cells of the testicle have this function?"

The testicle is well suited for such a study, both from its anatomic relations and from its histologic structure. It is easily accessible to experimental manipulations, and natural abnormal placements of the organ are not uncommon, entailing characteristic changes of structure which greatly aid in analyzing the possible functions of its several component elements.

Historical.—Despite the statements to the contrary by several writers (von Lenhossèk, 1897, Plato, 1896, Bouin et Ancel, 1903), Leydig (1850), and not Kölliker (1854), first described the presence of characteristic cells lying within the interstices of the seminiferous tubules. Kölliker's careful histological study of the interstitial cells appeared four years later. He characterized these structures as clear round cells, analogous to embryonic connective tissue, vacuolated, and containing fat and pigment granules. An important fact pointed out by Kölliker, and one too little considered in certain theories which have been advanced to explain the functions of the interstitial cells, is the constant presence of these cells not only in the interstices of the seminiferous tubules, but in the

* Received for publication, January 5, 1911.

mediastinum and connective tissue septa of the testis, and under the tunica albuginea.

The work of von Ebner (1871), confirmed and extended that of Leydig, Kölliker, Henle, and others. He described the interstitial cells as irregularly polygonal, the nucleus vesicular and sometimes double, the protoplasm granular and containing fat and pigment. Von Ebner agreed with Kölliker that the interstitial cells were of connective tissue origin and this has come to be the prevailing view with later writers. It is true that Hofmeister (1872) was inclined to view the interstitial cells as epithelial structures. He noted their great variability in various species and in the same species from time to time. He found in the fetus that their number diminished progressively until birth, and that after birth they were scantily present until the onset of puberty.

Von Hansemann (1895), studying the testicle of the hibernating marmot, found that spermatogenesis ceases during the hibernating period, and that during this season the interstitial cells almost completely disappear; with the resumption of spermatogenesis in the spring the interstitial cells reappear, and indeed in such quantity that upon section the testicle presents a sarcoma-like picture. He also pointed out that, whereas the sperm-forming cells disappear in patients suffering from febrile and wasting diseases (tuberculosis, cancer, etc.), the interstitial cells increase in number.

Reinke (1896), discovered in the testicle of an executed criminal the crystals which bear his name. He found them in the interstitial cells and lymphatics, and this led him to advance the tentative opinion that they represent the internal secretion of the testicle. Reinke's crystals have been found only in the human testicle.

The papers of Bouin et Ancel (1903, 1904) are the most important which have appeared since the work of Leydig and Kölliker. From a few experiments and a thorough study of normal and cryptorchid testes from various mammalia, they have concluded that the interstitial cells represent a definite organ, "la glande interstitielle," which furnishes an internal secretion to the body, and probably serves in addition as a trophic organ for the Sertoli and sperm-forming cells. They bring forward convinc-

ing evidence that the cells of Leydig are gland cells which in all probability furnish an internal secretion to the body. They do not, however, satisfactorily eliminate the Sertoli cell as a possible source of an internal secretion, merely stating that in animals with one testicle removed and the vas deferens on the other side ligated, the Sertoli cells show "des signes de dégénérescence," presumably fatty degeneration, since this is the only evidence of degeneration mentioned. They did not succeed in any case in causing the disappearance of the Sertoli cells from the seminal tubules, and nothing short of this can be admitted as morphological evidence. It will be shown later in this paper that the presence of an excessive amount of fat in the Sertoli cells is not a sign of degeneration, but rather an evidence of functional activity on the part of these cells. It should be insisted upon that as long as any cells with well preserved nuclei remain in the seminal tubules they can not be positively eliminated as possible producers of an internal secretion. The important work of Bouin and Ancel will be referred to hereafter, as well as the contributions of other writers, which can be more profitably reviewed in connection with the special parts of the subject of which they treat.

Embryology.—There is general agreement among recent investigators upon the mesenchymal origin of the interstitial cells. Whitehead's (1904) very clear description may be partially quoted. "The intertubular tissue of the testis of the pig embryo in stages immediately preceding the appearance of Leydig cells is a mesenchymal structure derived from the mesothelium of the genital ridge. Histologically, it is a connective tissue syncytium, consisting of cells and an exoplastic network of fibrils. The cells are scarcely more than naked nuclei, though some have a small collection of cytoplasm at one pole. From the cells of this tissue Leydig's cells are developed by growth of cytoplasm. At first they are markedly branched; some of the branches are connected with the general exoplastic network, so that the cells retain the syncytial arrangement of their ancestors. They increase in number and size very rapidly and soon lose their branches.

. . . . . .

"Leydig's cells pass through two phases of growth, between which a phase of atrophy intervenes. Growth is very rapid from their appearance in the embryo 2.4 centimeters long, until the length of 3.5 centimeters is reached. This is followed by the phase of atrophy, during which the cells return almost to their first state of nearly naked nuclei. This process reaches its acme in the embryo 14 centimeters long. Synchronous with it there is extensive growth of the seminal tubules, particularly in length, so that they are much convoluted, and the inter-tubular spaces are correspondingly narrowed. In the embryo 20 centimeters long, the cells enter upon the second phase of growth, which attains its maximum in the pig of 28 centimeters very near to term. Here the cells are enormously increased in size, so that they constitute the predominating feature of the microscopic picture."

During the period from birth to puberty, which may be regarded as the resting period of the testicle, the interstitial cells are scanty; at the time of puberty they again increase markedly in size and number.

Histology.—The microscopic appearance of the interstitial cells varies markedly in the same species from time to time, and in different species. They have been observed in all the classes of vertebrates, and analogous structures exist in the testes of invertebrates. They are found in all parts of the testicle except the epididymis, and are frequently very numerous just beneath the tunica albuginea. The capillaries of the testicle, on their way to supply the seminiferous tubules, are surrounded by interstitial cells. A striking analogy in this respect exists between the testicle and the adrenal, where the cortical cells surround the capillaries leading to the medulla. The interstitial cells vary greatly in size. They are irregularly polygonal in shape, frequently resembling liver cells or the cells of the corpus luteum. The nucleus, which may be double, lies eccentrically, and is surrounded by a condensed endoplasm which in turn merges peripherally into a thinner, vacuolated ectoplasm (plate XXX, Fig. 1). A certain small proportion of the cells are homogeneous throughout.

The foregoing description is true of material fixed and stained in the usual way, that is to say, without special effort to place in evi-

dence the granular structure of the protoplasm (Altmann's granules). If, however, small pieces of testicle be fixed by the method of Altmann (Schmorl, 1909), or some of its modifications (Benda, Schridde), methods which depend essentially upon the use of oxidizing agents such as potassium dichromate or osmium tetroxide, and then stained according to the methods of Altmann, Benda, or Weigert (Fauré-Fremiet, Mayer, Schaeffer, 1910), one obtains an entirely different protoplasmic picture from that which has become classical. It is now seen that the dense endoplasm around the nucleus is composed of closely packed granules of various sizes (plate XXXI, Fig. 2), and that the relatively clear ectoplasm contains fewer granules. In the ectoplasm a second kind of granule is seen, which, I think, is identical with certain granules described by Whitehead (1906) in the ectoplasm of the interstitial cells. With formol fixation and paraffin sections one can stain these granules with methylene blue. With the acid fuchsin of Altmann they stain a distinctly clearer red than the other cell granules (Fig. 2). They usually lie in a small vacuole. Treated by Weigert's myelin-staining method, or Heidenhain's iron-hematoxylin they are colored black.

Fat is quantitatively a very variable constituent of the interstitial cell, though rarely entirely absent. In human beings and in some animals (dog and cat) it is present in relatively large amount, whereas in the interstitial cells of the pig, fat droplets are usually very scanty. In discussing the functions of the interstitial cells we shall have occasion to consider further the significance of their fat content.

Pigments, soluble and insoluble, are common constituents of the interstitial cells of many animals. The testicle of the pig is colored brown by them, and they are in part soluble in water. Other pigments are present as lipochromes—pigmented complexes of a lipoid nature. Nothing is known of the structure or possible functions of the pigments of the testicle. Crystals or crystalloid substances, such as those described in the human testicle by Reinke and Lubarsch, seem to be very rare in the testicles of the lower mammalia.

THE FUNCTIONS OF THE INTERSTITIAL AND SERTOLI CELLS OF THE TESTIS.

It is one of the oldest and most commonly observed phenomena that castration of the young animal profoundly alters the normal growth and development of the body (Tandler and Gross, 1907, 1908, Griffiths, 1895). We speak of the organs of generation as primary sexual characters, for they differentiate the sexes in early embryonic life. With the maturation of the sexual life of the individual, other characters become evident which further separate the sexes. Thus at puberty the larynx grows rapidly and the voice changes, the growth of hair increases upon characteristic areas, the external genitalia develop more rapidly, the sexual instinct becomes manifest, skeletal differences develop in the male and female—in a word, under the influence of some new and powerful stimulus the body proceeds to sexual maturation. All these delayed manifestations of sex are grouped together as secondary sexual characters.

Whether or not the primary sexual characters of the male are guided in their development by a testicular secretion, remains to be proven. Their origin, at any rate, would seem to rest upon somatic inheritance. It is certain, on the other hand, that secondary sexual characters depend entirely upon some function of the testis, for castration prevents their appearance. The problem is not, then, whether the testes furnish a secretion essential to the normal development of the body, but what element of the testicle has this function, and what the nature of the secretion really is.

The testicle is a glandular structure, one function of which is the secretion of sperm. It contains three parenchymatous elements; namely, the series of sperm-forming cells (spermatogonia, spermatocytes, and spermatids), the Sertoli cells, and the interstitial cells. Which of these three elements is responsible for the production of a secretion which governs the development of secondary sexual characters?

A study of the condition of cryptorchidism will partially elucidate the problem. The material for such a study may be obtained very readily from slaughter-houses, and the advantages of such material

as compared with the difficult study of the rare condition of cryptorchidism in man, or in cryptorchid animals experimentally produced in the laboratory, will be easily appreciated. It is the custom of stock raisers to castrate male pigs at an early age. At the time of castration, in the great majority of the animals, both testicles have descended and are consequently excised. Occasionally one testicle descends before the other, and in such cases only one organ is removed, the other descending later. The condition of permanently retained testes, cryptochidism, is not uncommon in the pig, and may be unilateral or bilateral. Finally, a certain number of normal animals with two scrotal testes come to slaughter. An almost unlimited amount of healthy experimental material is thus available, Nature and the stock-raiser relieving us here of the drudgery and uncertainties of laboratory experimentation.

In the present study I have examined fifty-six cryptorchid testes from the pig, seven from the sheep, and six from man. In no single instance have I found spermatozoa, and only in very young cryptorchid pig testes have spermatogonia been demonstrable. This is in accordance with the well established clinical fact that bilaterally cryptorchid animals are invariably sterile (Bouin et Ancel, 1904, and Whitehead, 1908). Conflicting statements upon this point are met with, but in a careful search of the literature no well authenticated case of a fertile cryptorchid has been found, whereas most writers agree upon the infertility of doubly cryptorchid animals. It should be borne in mind that a truly cryptorchid testicle lies completely within the internal abdominal ring; confusion has arisen through the lax application of the term to funicular and incompletely descended scrotal testes.

All writers agree that the doubly cryptorchid animal develops every attribute of the normal male, save the power of propagation. Secondary sexual characters are well developed and the male passion is normally or even excessively evident (Whitehead, 1908, Bouin et Ancel, 1904). Sections from the cryptorchid pig testicle reveal a truly remarkable picture, due to the excessive number of Leydig cells present. One observes that whereas in the normal organ (plate XXXII, Fig. 3) the spermatic tubules greatly predominate

over the interstitial cells, in the cryptorchid testicle the reverse is true (plate XXXII, Fig. 4). The interstitial cells of the cryptorchid testis are usually larger than those of the normal organ, but otherwise their histological structure is the same. The seminiferous tubules, on the contrary, present marked abnormalities. The basement membrane of the tubule is thickened, and it is lined only by a single layer of Sertoli cells (plate XXXIII, Fig. 5). *No trace whatsoever of sperm-forming cells can be found in the adult cryptorchid testicle.* In very young cryptorchids, large clear primary sperm cells (Ursamenzellen) are seen (plate XXXIII, Fig. 6), but they apparently do not develop further, and soon disappear.

If one compares now, as a useful criterion of the development of secondary sexual characters, the external genitalia of normal, castrated, and cryptorchid pigs, one finds that whereas the genitalia of castrated animals are markedly undeveloped and atrophic in appearance (plate XXXIV, Fig. 7), no distinction whatever can be made between the genitalia of normal (plate XXXIV, Fig. 8) and cryptorchid animals (plate XXXIV, Fig. 9). Since, then, in the absence of the sperm-forming cells secondary sexual characters develop quite normally, one may safely eliminate these cells from the problem of determining which element of the testicle furnishes an internal secretion to the organism.

It is unfortunately not so easy to eliminate similarly either one or the other of the two remaining parenchymatous elements, interstitial cells and Sertoli cells. No method has yet been found which will cause the disappearance of one while leaving intact the other. Various methods have been tried, such as occlusion of the vas deferens (Bouin et Ancel, 1904, Griffiths, 1895), x-ray injury to the testicle (Simmonds, 1907, Herxheimer and Hoffmann, 1908), freezing (Cevalotto, 1909), etc., but, whereas the spermatogenic cells disappear after any sufficient injury to the testicle, the Sertoli and interstitial cells persist. Artificially produced cryptorchid testes in dogs present essentially the same histologic picture as that described above in cryptorchid pigs; both Sertoli and interstitial cells persist, the sperm-cells disappearing. I have successfully transplanted pieces of testicle under the rectus muscle of castrated dogs (auto-transplants), but here too the Sertoli cells and

the interstitial cells seem equally viable, both persisting in the transplant (plate XXXV, Fig. 10). I have not been able to cause the disappearance of the Sertoli or interstitial cells in dogs by ligating the vasa deferentia. Such ligations in young dogs do not prevent the normal maturing of the sperm-cells, but subsequently these disappear.

Confronted, then, with the seeming impossibility of solving the question in hand by the direct method of physically eliminating either the Sertoli or the interstitial cells, after the manner in which the spermatogenic cells are eliminated in the cryptorchid testicle, one is forced to adopt the more usual method of determining, from a study of the facts obtainable, which of two possibilities is the better supported by these facts. We may first examine the evidence for and against the interstitial cell as the elaborator of an internal secretion, and then seek to establish from the facts at hand the true function of the Sertoli cells.

We have seen that embryologically the interstitial cell is of mesenchymal origin. Histologically it presents the type of a gland cell—a plentiful cytoplasm rich in cell granules. The histological resemblance of the interstitial cells to the cells of the liver is worthy of emphasis, and especially is this true when both are stained to demonstrate their rich content of cell granules. The interstitial cells stand in the closest possible relation to the rich capillary meshwork of the testicle; they surround the capillaries in their course to the tubules, and are bathed in the large inter-tubular lymph spaces.

A compensatory hypertrophy can be demonstrated in the interstitial cells under certain conditions. In a pig from which one testicle has been removed at an early age, the interstitial cells of the remaining organ are present in markedly increased numbers (plate XXXV, Fig. 11). So striking is this increase that one can easily tell microscopically the monoscrotal from the biscrotal organ (Bouin et Ancel, 1903). But a more convincing proof of such compensatory hypertrophy is seen on comparing the monocryptorchid testicle (plate XXXVI, Fig. 12) of an animal in which the other organ has descended and been removed, with the testes of a bilaterally cryptorchid animal (plate XXXVI, Fig. 13). In the former the predominance of the interstitial cells over the atrophic seminifer-

ous tubules is much more striking than in the latter. At the same time no hypertrophy of the Sertoli cells can be noted. Indeed they are present in relatively far smaller numbers than in the normal organ. If the Sertoli cells really function as producers of an internal secretion, one would certainly expect to find evidence of their increase in monocryptorchid testes, as well as in animals possessing only one scrotal testis, especially in view of the striking augmentation of the interstitial cells under these conditions.

It has been suggested by Plato (1896) that the interstitial cells have for their function the furnishing of nutriment, in the form of fat, to the Sertoli and spermatogenic cells. Quite apart from a total lack of evidence in support of such a view, Plato's claim to have demonstrated the passage of droplets of neutral fat from the interstitial into the Sertoli cells through certain preformed canaliculi of the tubular wall has been thoroughly discredited (Beissner, 1898). Moreover, the conditions existent in the cryptorchid testis remove any doubts of its falsity, for here we find an enormous relative increase of the interstitial cells, with atrophic seminiferous tubules and a complete absence of spermatogenic cells.

Von Lenhossèk (1897) advanced the view that the interstitial cells furnish a pabulum for the use of the cells of the seminiferous tubules. The discussion of such a theory is profitless, based as it is on slight and equivocal evidence. The same may be said of Harvey's (1875) belief that the interstitial cells represent nerve cells.

Von Bardeleben (1897) noticed a fancied resemblance between the Sertoli and Leydig cells, and thought the "worn-out Sertoli cells" were in time replaced by young Leydig cells. Such theories are, in the main, only of minor historical interest, but Goldmann (1909), using the vital stains of Ehrlich and Mesnil, has recently revived interest in the view of von Bardeleben by claiming the demonstrable passage of Leydig cells through the walls of the seminiferous tubules, and indeed he figures quite beautifully such an event. I have repeated with care the work of Goldmann and can say that in the course of an enumeration of 1,000 Leydig cells in sections from the testicles of rats which had been vitally stained with trypan-blue and pyrrol-blue, no suggestion of the phe-

nomenon described by Goldmann was observed. It must be very rare, for among the many careful histological studies by various observers of the easily stainable Leydig cells, no one has described a similar picture.

In cryptorchid animals, the findings, both in the testicles and in the external genitalia, demonstrate clearly enough that the interstitial cells represent a structure which has a general bodily function, and is not in any reasonably thinkable way connected with the nutrition of the spermatogenic cells.

Such a nutritive organ does exist, however, within the seminiferous tubule in the form of the very specialized Sertoli cells. The greatest divergence of opinion has existed in regard to the function of these cells since their discovery by Sertoli in 1865. This is evident from the various names applied to them by different investigators, such as "sustentacular cells" (F. Merkel), "follicle cells" (La Valette St. George), "Nährzellen" (Peter), and "sperm-nourishing-cells" (Grobben). The Sertoli cells form a single-layered lining for the membrana propria of the seminiferous tubules. The cell boundaries are indistinguishable in ordinary preparations, and Regaud (1910) describes them as forming a syncytium. Von Ebner (1902) states, however, that in physiological salt solution they can be isolated as discrete cells. In the non-functioning tubule they assume the form of cylindrical epithelial cells, but during spermatogenesis, filmy protoplasmic processes reach far out into the lumen of the tubule. Their cytoplasm always contains fat droplets in very considerable amount, a finding to be described in more detail presently, and this fat appears in the Sertoli cells before the embryonic differentiation of the cells of Leydig (Whitehead, 1904, Allen, 1904).

A brief review at this point of the process of spermatogenesis will facilitate an understanding of the functions of the cells of Sertoli. Lying partially embedded in the protoplasm of the Sertoli cells, close to the basement membrane of the tubule, are small round cells with a more or less richly chromatic nucleus. These are the sperm-mother cells or spermatogonia. They initiate a generation of sperm by dividing into two cells, known as spermatocytes of the

first order, which in turn undergo mitotic division, giving rise eventually to four spermatids. Shortly after the formation of the spermatids, the old generation of spermatozoa are cast off into the lumen of the seminiferous tubule, and now the spermatids and Sertoli cells enact a scene which is quite without analogy elsewhere in the body. A group of spermatids (eight to twelve in the rat), form a true protoplasmic union with the cytoplasmic prolongation of a Sertoli cell, so that together they present the picture of an axially elongated, multinucleated protoplasmic mass.

During this development of the spermatids from the spermatogonia, and their subsequent conjugation with the Sertoli cells, important changes have taken place in the fats in the Sertoli cells. Von Ebner (1902) has described a "circulation" of the fat of the Sertoli cell, during the course of a spermatic generation, from the base of the Sertoli cell toward the lumen of the tubule, and noted that *pari passu* with the development of the sperm, the fat diminished. He concluded that it was utilized as nutriment for the developing spermatozoa. His clear and convincing descriptions are based upon histological changes in the normal testes, but no evidence of a physiological nature has, so far as I am aware, been brought forward in its support. Such evidence, obtained from a microchemical and chemical study of normal and cryptorchid testes, is presented elsewhere in this journal (Hanes and Rosenbloom, page 355) and may be summarized very briefly at this point. If seminal tubules in various stages of spermatogenesis are stained for fat, it will be seen that as spermatogenesis proceeds the large fat droplets which are present in the basal portions of the Sertoli cells in the beginning, divide into very much smaller droplets and pass centralward in the protoplasmic prolongations of the Sertoli cells, and thence into the spermatids after their union with the Sertoli cells. During its migration the fat can be shown by appropriate staining methods to have changed in its chemical character from a neutral fat to a lipoid. Using Weigert's method for the staining of myelin, Regaud (1910) has also demonstrated lipoids in the Sertoli cells and spermatids. The exact manner in which the fat furnished by the Sertoli cells is eventually utilized by the spermatids and sperm, shares the obscurity which envelops the question of the ultimate

metabolism of fats by the cells throughout the body. In cryptorchid testes it has been pointed out that the sperm-forming cells have completely disappeared (Fig. 5). If the fat of the Sertoli cells is destined for the nutriment of the spermatids and sperm, the absence from the seminal tubules of these elements should result in the presence of an excessive amount of fat in the Sertoli cells. That such is strikingly the case will be seen from a section of a cryptorchid testis stained to demonstrate fat (Figs. 4 and 5). The Sertoli cells are seen to be loaded with large neutral-fat droplets. Upon chemical analysis of normal and cryptorchid testes, the microchemical findings are corroborated. Of the dried weight of normal pig testes, 19 per cent. was found to be fatty material, whereas the percentage of fatty substances present in cryptorchid testes was found to amount to 31 per cent. of their dried weight. In other words, the fat content of the cryptorchid testicle is almost double that of the normal. We conclude, therefore, that fat accumulates in the Sertoli cells of the cryptorchid testis because of the absence of the spermatogenic cells which normally utilize this fat.

The classical researches of Miescher (Burian, 1906) upon salmon sperm, and upon the sperm of the bull, fully support the above conclusion. He found that, whereas the head or nucleus of the sperm is almost devoid of fatty matter (1 to 2 per cent.), the tail, or cytoplasm, is extremely rich in fatty substances, containing 58 per cent. of ether-soluble material, as compared with 42 per cent. of protein. Upon analysis of the 58 per cent. of fatty matter of salmon sperm, 50.4 to 53 per cent. was found to be lecithin; 17 per cent., cholesterin; and 31.3 to 35.4 per cent., true fat, very rich in oleic acid. Very similar results were obtained from analyses of bull spermatozoa (Miescher), and the sperm of the herring (Matthews).

The general state of nutrition of the body has no demonstrable bearing upon the fats of the testicle. It is impossible to cause their disappearance by starvation, and patients dying from wasting diseases with extreme cachexia show no diminution in the testicular fat (Cordes, 1898, Thaler, 1904, Traina, 1904).

From the facts at hand it may be concluded that one function of the Sertoli cell is the providing of nutriment to the peculiarly situated spermatogenic cells. Histological, chemical, and physiological

evidence, amply support this conclusion. Whether or not the Sertoli cells furnish in addition an internal secretion to the body, we cannot say with absolute certainty. There is no evidence whatever that they elaborate an internal secretion, whereas an abundance of facts points to their functioning solely as sperm-nourishing cells. The interstitial cells of Leydig, on the other hand, present the characters of active gland cells, and the evidence which I have presented has lead me to believe that they furnish an internal secretion to the male body necessary for its normal development.

CONCLUSIONS.

1. The mammalian testicle elaborates an internal secretion necessary to the normal development of secondary sexual characters.

2. The spermatogenic cells play no part in the formation of this secretion.

3. One function of the Sertoli cells is to supply fat to the developing sperm-cells. In the absence of the sperm-cells from the mammalian testis, fat accumulates in large amount in the Sertoli cells. The Sertoli cells seem to take no part in the production of an internal secretion.

4. The interstitial cells of Leydig are, in all probability, responsible for the production of the internal secretion of the mammalian testis.

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EXPLANATION OF PLATES.

Plate XXX.

Fig. 1. × 1,000. Zenker's fluid, hematoxylin and eosin. Section of cryptorchid pig testicle showing the interstitial cells surrounding a capillary. Some of the cells are homogeneous throughout, but most of them show a dense endoplasm and a vacuolated peripheral ectoplasm containing definite granules.

Plate XXXI.

Fig. 2. × 1,000. Altmann's stain. The granules of the cells are stained red, fat droplets black. As in Fig. 1, the endoplasm and the ectoplasm are sharply contrasted, due to their granular contents.

Plate XXXII.

Figs. 3 and 4 are microphotographs taken under the same magnification. In the normal testicle (Fig. 3) the actively functioning seminal tubules dominate the picture; the interstitial cells occupy the interstices of the nearly contiguous tubules. In the cryptorchid testicle (Fig. 4) the picture is exactly the reverse; the seminal tubules are shrunken, and the interstitial cells greatly predominate. Both sections are stained to show fat (Sudan III). The round droplets of fat form a peripheral fringe in the normal seminal tubules (Fig. 3), while in the tubules of the cryptorchid testis (Fig. 4), the fat droplets are very abundant, filling the Sertoli cells.

Plate XXXIII.

Fig. 5. High power drawing of two seminal tubules of a cryptorchid pig testis, surrounded by interstitial cells. The basement membranes of the seminal tubules are thickened, and they are lined by Sertoli cells which contain large fat droplets. The interstitial cells appear normal in every way, contrasting sharply with the obviously atrophic, aspermatic seminal tubules.

Fig. 6. High power drawing of a section of a single seminal tubule from a very young cryptorchid pig testis. The tubule is lined by Sertoli cells containing large fat droplets (shown as vacuoles in the drawing). The primordial sperm cells (Ursamenzellen) with deeply staining nuclei lie nearer the lumen.

Plate XXXIV.

Fig. 7. Photograph of the urino-genital apparatus of an adult castrated pig.

Fig. 8. The urino-genital organs of a normal pig.

Fig. 9. The urino-genital organs of a monocryptorchid pig (the other testicle had descended normally and had been removed).

The photographs are taken to scale and show the bladder, prostate, seminal vesicles, glands of Cowper, and penis. The organs of the castrated pig (Fig. 7) are small and atrophic, whereas the organs of the cryptorchid pig (Fig. 9) are as well developed as in the normal animal (Fig. 8).

Plate XXXV.

Fig. 10. Section from a piece of dog's testicle transplanted under the rectus muscle. The spermatogenic cells have disappeared from the seminal tubules, leaving only the Sertoli cells. Many interstitial cells have survived.

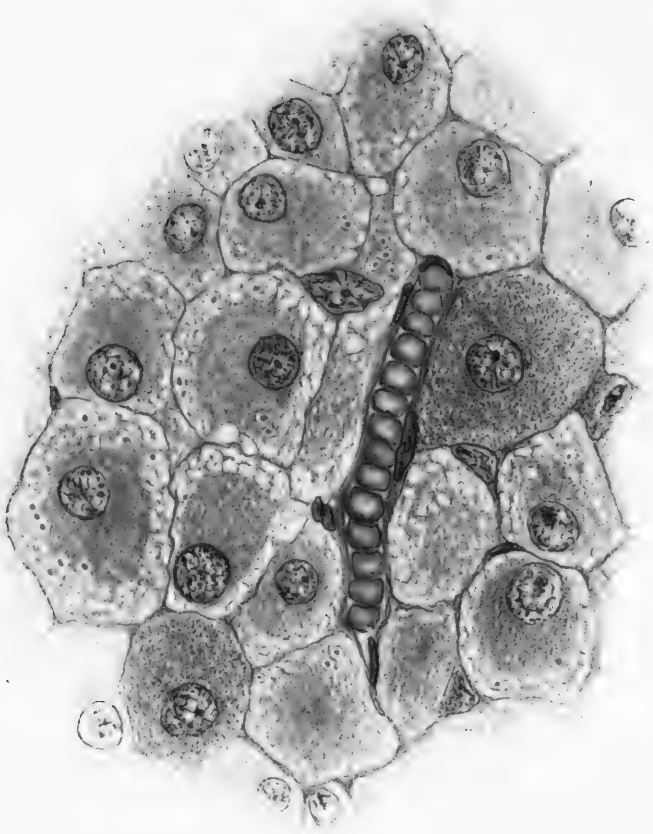
Fig. 11. Section from the normal testicle of a pig having only one scrotal testis, the other having been removed in early life. This figure is to be compared with Fig. 3, which represents the normal relation of seminal tubules and interstitial cells in pigs having two scrotal testes.

Both sections have the same magnification. In Fig. 11 the relative increase of the interstitial cells is well seen (compensatory hypertrophy).

Plate XXXVI.

Fig. 12 shows the relative proportion of interstitial cells to seminal tubules in a pig having only one cryptorchid testis and no scrotal testis.

Fig. 13 shows the same in a pig having two cryptorchid testes. In Fig. 12 the interstitial cells are relatively far more abundant than in Fig. 13 (compensatory hypertrophy).



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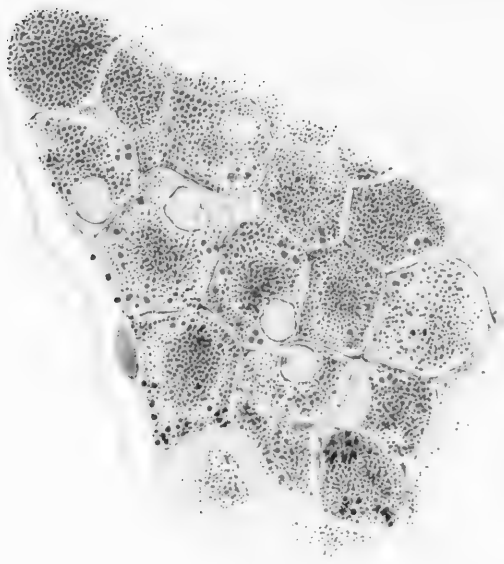
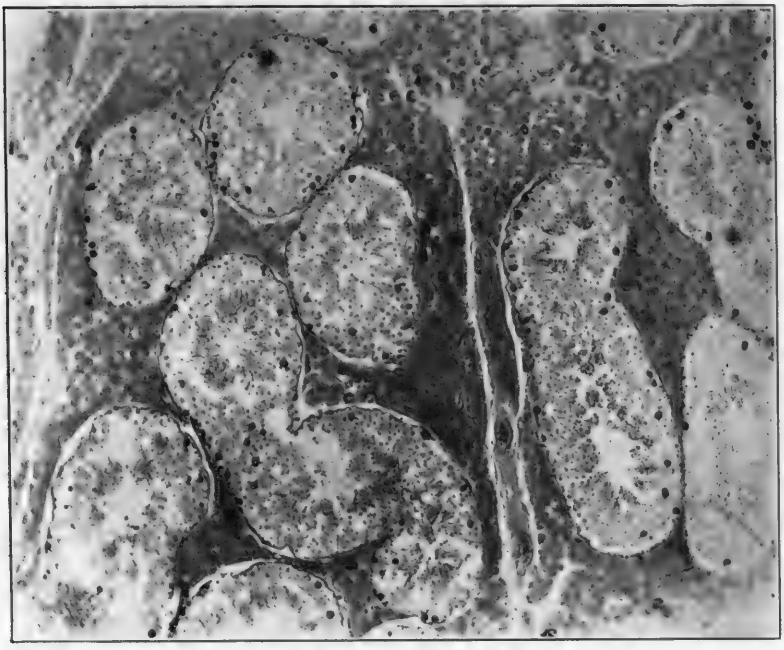


FIG. 2.



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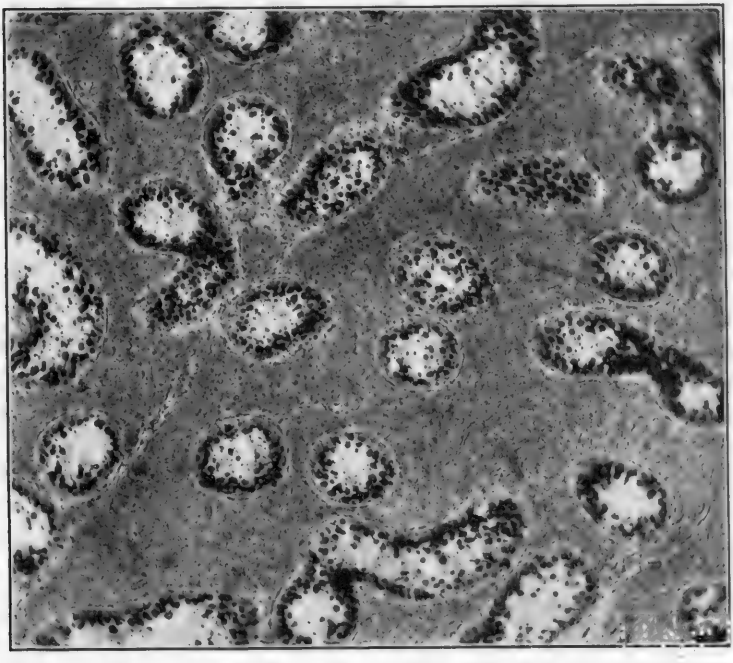

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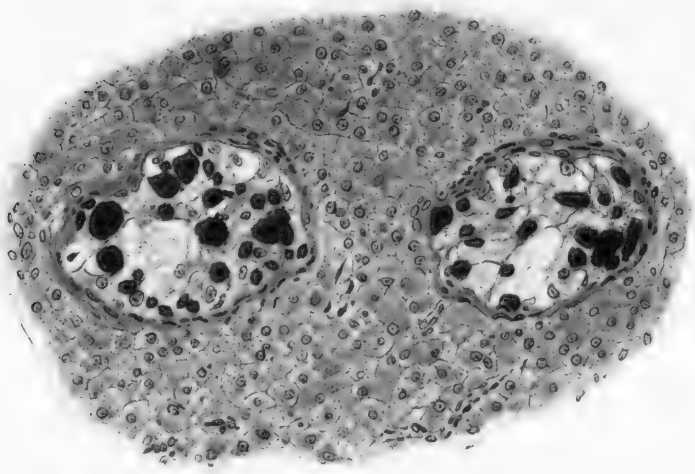

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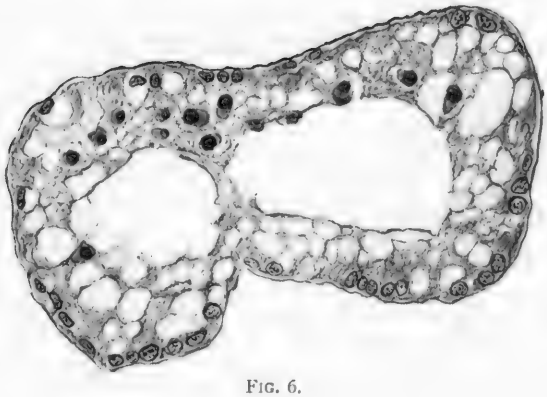

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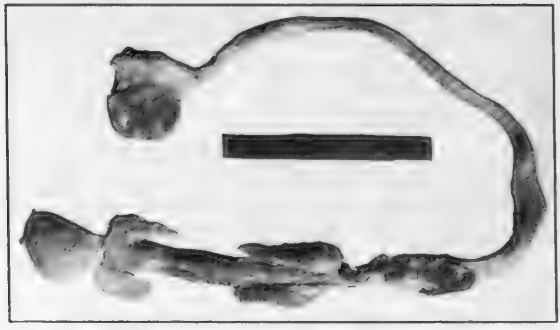

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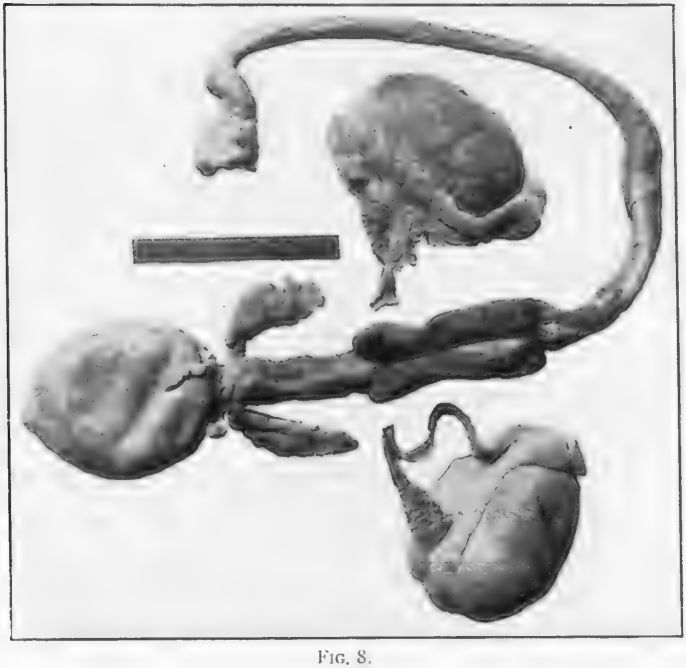

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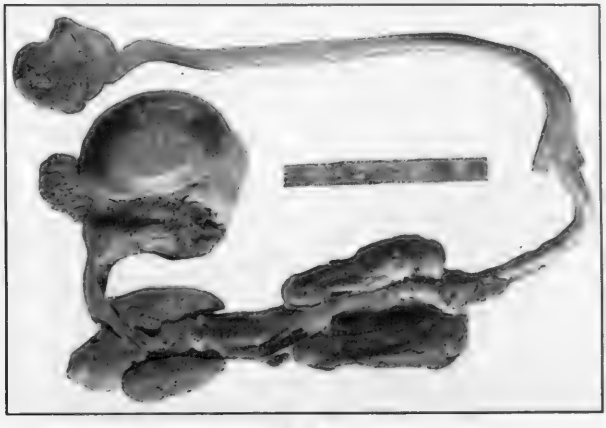

FIG. 9.

FIG. 10.

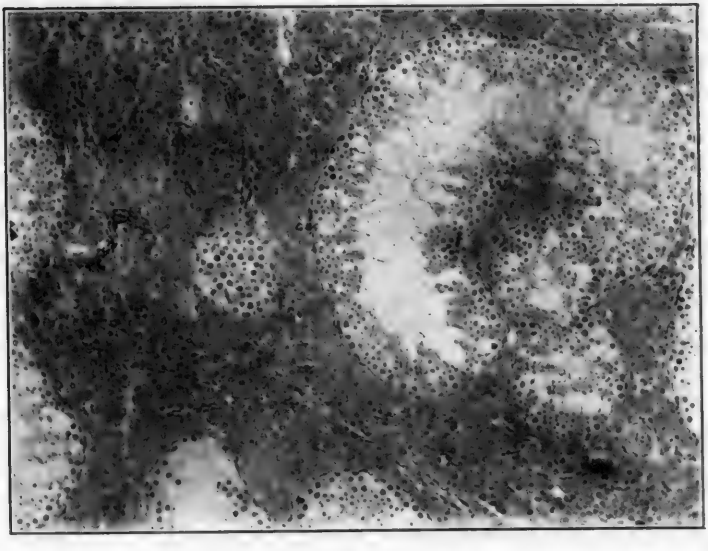

FIG. 11.

FIG. 12.

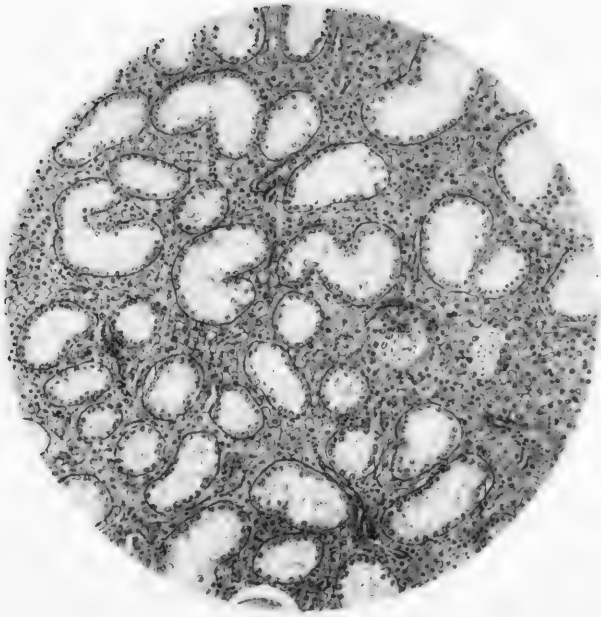

FIG. 13.

A HISTOLOGICAL AND CHEMICAL STUDY OF THE FATTY MATTER OF NORMAL AND CRYPTORCHID TESTES.*

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PLATE XXXVII.

I. HISTOLOGICAL OBSERVATIONS.

In the course of an investigation of the interstitial cells of Leydig in the testes of various mammalia, our attention was early directed to the large amount of microchemically demonstrable fat in the Sertoli and spermatogenic cells of the seminal tubules. This observation is by no means new. Kölliker (1), in 1854, described similar findings, and all subsequent writers upon the subject have emphasized the point (2). From a study, however, of a large number of cryptorchid testes from the pig, we have accumulated additional data which bear so directly upon the problem of the metabolic rôle played by the fats of the seminal tubules of the testis, that the subject seems worthy of detailed analysis.

Within the seminal tubules there are two very distinct histological elements, the sperm-forming cells, and the Sertoli, or sperm-nourishing cells. These latter cells line the inner surface of the seminal tubules in a syncytium-like manner, and the former lie partially embedded in their cytoplasm. At the beginning of a spermatic generation large droplets of fat are constantly present in the basal portions of the Sertoli cells, so that a section of such a tubule stained with Sudan III, or osmic acid, shows a peripheral ring of fat droplets (plate XXXVII, Fig. 1). These large droplets respond to all the tests for neutral fats. As spermatogenesis proceeds, however, these large droplets break into smaller ones, and, leaving the basal portions of the Sertoli cells, they pass into the central protoplasmic

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prolongations of these cells. The spermatids, having now in the course of spermatogenesis formed a true protoplasmic union with the Sertoli cells, the small droplets of fat are seen to have partially passed into the spermatids (von Ebner). In a section of a seminal tubule at this stage of spermatogenesis (stained by osmic acid after having been previously treated with potassium dichromate—Müller's fluid, twenty-four to forty-eight hours), it is found that the small central droplets of fat, although continuing to stain with Sudan III, will no longer reduce osmic acid and stain black. The large droplets of fat in the same and neighboring tubules still stain intensely black with osmic acid. Obviously then, the fat of the Sertoli cells, as it passes toward the lumen of the seminal tubules, undergoes some chemical change. The chemical treatment just described (chromatization followed by osmic acid) is essentially an application of Marchi's stain to the fats of the testis, and Marchi's stain, as is well known, is employed to differentiate fats from the peculiar lipoid complex known as myelin. We have concluded, therefore, that the change in the neutral fats of the Sertoli cells represents a transformation to a lipoid. This conclusion is supported by the work of Regaud (3) who, using Weigert's method for staining myelin, has also demonstrated lipoids in the Sertoli and spermatogenic cells.

A discussion of some of the physical and chemical properties of the myelin bodies will be found in a note at the end of this paper, and will not be entered upon at this time. Regaud (3) has sought to establish a relation between the fats of the Sertoli cells and their cell granules or mitochondria (Benda (4)). To pursue this question would involve a discussion of the very difficult and little understood problem of the structure and functions of cell granules, and it must therefore be abandoned for the present. We may safely conclude with von Ebner, however, that histologically fat seems to be an important constituent of the nourishment furnished by the Sertoli cells to the rapidly maturing sperm-cells.

Physiological evidence of the important rôle played by the fats in spermatogenesis has been wanting. Such evidence is obtainable, we think, from a study of the cryptorchid testes of the pig. In

a section of a cryptorchid testis (plate XXXVII, Fig. 2), one sees, in addition to an enormous increase of the Leydig cells, that the seminal tubules are much smaller than normally, and are lined only by a single layer of Sertoli cells. *The entire series of spermatogenic cells has completely disappeared,* a finding which is in harmony with the well-known sterility of double cryptorchid animals (Ancel and Bouin (5)). A section of such a cryptorchid testis, stained for fat, shows a truly remarkable quantity of large fat droplets in the Sertoli cells (Fig. 2). Indeed, the protoplasm of these cells is often so nearly replaced by fat that they resemble fat cells. This fat reacts chemically in every way like the neutral fats found elsewhere in the body.

The histological evidence of the presence of an excessive amount of fat in the seminal tubules of cryptorchid testes is strikingly substantiated by chemical analysis. The results tabulated on page 359 show that the fat content of the cryptorchid testis is approximately double that of the normal organ. We have seen that fat is apparently actively employed by the Sertoli cell as a metabolic element in the course of its activity as a nourisher of the sperm-forming cells. But in the cryptorchid testis these latter cells are lacking. We have concluded, therefore, that the large accumulation of fat found in the Sertoli cells of cryptorchid testes represents the fat which in the normal testis is utilized in the course of spermatogenesis. The Sertoli cell is endowed with the function of accumulating the fats needed during the process of spermatogenesis, and even in the absence of the spermatogenic cells, which normally utilize this fat, the Sertoli cell continues to perform its function. The presistence of such specialized cell functions under the most abnormal conditions is among the best recognized of biological phenomena.

There was a time, not far past, when such accumulations of fat as those just described in the Sertoli cells of the cryptorchid testis, were simply labelled "fatty degeneration" and set aside. Under the influence of recent pathological and biochemical research upon normal and abnormal fat metabolism, Virchow's time-honored phrase has lost most of its charm. There exists to-day no acceptable evidence that protoplasm ever "degenerates" into fat (Rosenfeld

(6)). We are beginning to see more clearly that the presence of abnormal quantities of neutral fats in the body cells is an expression of metabolic derangement, and not necessarily of protoplasmic degeneration. The procuring of foodstuffs, as represented, for example, by the fats, and their subsequent utilization by the cell, represent, it would seem, separately specialized functions of the cell. Normally these functions are beautifully coördinated, so that intake and utilization balance, but this coördination may be disturbed, as seen, for example, after toxic injury to the cells of the liver, with the consequence that fat continues to accumulate in the cell but is no longer metabolized with normal rapidity. The end result of this derangement of cell functions is now termed "fatty infiltration," and the idea suggested by the term is well supported by the work of Rosenfeld and others. The abnormal situation of the cryptorchid testicle is accompanied, for some unknown reason, by a loss of the spermatogenic cells, which normally utilize in their growth the fats furnished by the Sertoli cells. Fat accumulates, therefore, in the Sertoli cells, and we may properly regard the process as that of fatty infiltration, and not fatty degeneration. The fat content of the testicle does not show variations, due to changes in the general condition of the body, exhibited by the stored-up neutral fats elsewhere. Extreme cachexia occasions no diminution in the testicular fat, nor does obesity cause an increase (Thaler (7), Cordes (8), Traina (9)).

The histological study of the fats of normal and cryptorchid testes revealed facts which led us to make a thorough chemical study of the subject. Our data in this connection are recorded below.

II. CHEMICAL DATA.

Desiccation of the Testes.—The testes within three hours after their removal from the body were cleaned from the epididymis, weighed, and run through a hashing machine. 1,005 grams of normal pig testicles were used for this study and 610 grams of cryptorchid pig testicles. Three separate amounts of each kind of material, obtained on different days, were employed, thus allowing for unavoidable variations in the testes, due to slightly varying ages of the pigs. The hashed organs were spread on glass trays and dried in a stream of warm air, with repeated mixings of the tissue. At the end of twenty-four hours the desiccated material was ground in a mortar, passed through a grinding machine, and dried *in vacuo* over sulphuric acid until of constant weight. The dried tissue

was reground to a fine powder and samples then taken for the extraction of the fatty matters.

Extraction of the Fatty Matter.—The extractions were carried out in the ordinary form of Soxhlet extractor and each extraction continued until a fresh supply of solvent yielded no residue on evaporation. Four thorough extractions of each sample of material were made successively with the following solvents: ether, alcohol, ether. The residue was then subjected to peptic digestion, after which the material was again thoroughly extracted with ether (Dormeyer (10)).

Chemical Analyses.—In order to detect any variations in the character of the fatty material contained in the normal testes as compared with that from cryptorchid testes, the material from each extract was examined separately. The separation of the phosphorized fat (lecithans) was carried out according to the method described by Koch (11). The phosphorus oxidations were made by the Neumann method (12), and the phosphoric acid weighed as magnesium pyro-phosphate.

Results.—The following table exhibits the results obtained. The data show that:

1. The proportion of fatty material in the cryptorchid pig's testis which is soluble in alcohol and ether is approximately twice that in the normal organ.

2. The percentage of phosphorized fats (lecithans) in the cryptorchid testis is likewise nearly double that in the normal glands.

3. The fatty materials in the cryptorchid and in normal testes which are soluble in alcohol and ether are nearly identical in their iodine numbers and saponification values (table I).

TABLE I.

Normal testes (1,005 grams); 84.36 per cent. water.

No.	Weight of dry tissue in grams.	1st extract: (in ether) grams.	2d extract: (in alcohol) grams.	3d extract: (in ether) grams.	(Dormeyer) 4th extract: (in ether) grams.	Total fat in grams.	Percentage of fats in dry tissue.	Percentage of fats in fresh tissue (average).
I	59.3545	7.2515	3.7450	0.0460	0.1620	11.2045	18.87	
II	49.1740	5.6020	3.3510	0.0245	0.1995	9.1770	18.66	2.97
III	48.9445	5.9600	3.3230	0.0010	0.2080	9.4920	19.38	

Cryptorchid testes (610 grams); 80.37 per cent. water.

IV	36.9345	7.6080	3.2435	0.0155	0.2180	11.0850	30.01	
V	37.2665	8.1175	3.2040	0.0155	0.2860	11.6230	31.19	6.01
VI	44.7222	9.8945	3.6175	0.0310	0.3775	13.9305	31.15	

Normal testes.

No.	Percentage of lecithans in dry tissue.			Percentage of lecithans in fresh tissue (average).	Saponification value.*			Iodine number† (Hübl's method).		
	Ether soluble.	Alcohol soluble.	Ether and alcohol soluble (average).		Ether soluble.	Alcohol soluble.	Ether and alcohol soluble (average).	Ether soluble.	Alcohol soluble.	Ether and alcohol soluble (average).
I	34.18	30.26	32.22	0.988	205.5	156.9	181.2	103.8	51.8	77.8
II	27.40	28.42	27.91		206.0	157.3	181.6	104.0	54.2	79.1
III	30.86	30.58	30.72		205.1	157.8	181.4	107.3	53.7	80.5
Cryptorchid testes.										
IV	42.27	28.27	35.27	1.75	226.5	164.2	195.3	106.0	53.2	79.6
V	42.08	21.22	31.65		226.4	165.1	195.7	106.1	53.8	79.9
VI	34.01	38.06	36.03		225.8	163.6	194.7	106.4	53.6	80.0

III. CONCLUSIONS.

1. About 19 per cent. of the dry weight of the normal pig testicle is fatty matter. Histologically this fat occurs largely in the cells of the seminal tubules, and especially in the Sertoli cells.

2. During spermatogenesis the fat of the Sertoli cell passes inward for the nutrition of the spermatids and spermatozoa. During this passage its character is altered from a neutral fat to a lipoid.

3. About 30 per cent. of the dry weight of the cryptorchid pig testicle is fatty material. Histologically this fat lies within the seminal tubules, partially filling the Sertoli cells. The spermatogenic cells have completely disappeared.

4. We conclude that the presence of such an excessive amount of fat in the cryptorchid testicle is due to the absence of the spermatogenic cells which normally utilize during their development the fat furnished by the Sertoli cells.

* The saponification value of a fat is the number of milligrams of potassium hydroxide that are neutralized by the fatty acids from one gram of the fat. It is a measure of the mean molecular weight of the fatty acids entering into the composition of a fat.

† The iodine value of a fat is the amount of halogen (computed as iodine) expressed in per cent. of the weight of fat that the unsaturated acid radicals it contains will take up. The saturated acids and their glycerides absorb no iodine and, therefore, the iodine value of a fat or a mixture of fatty acids is an index of the per cent. of saturated and unsaturated acids present. The acids with unsaturated linkages in more than one place absorb proportionally more iodine. The saponification and iodine values in the above table represent the mean of closely agreeing triplicate determinations.

IV. A NOTE ON THE MICROCHEMISTRY OF FATTY SUBSTANCES.

Recent studies in the microchemistry of the fatty substances have materially increased our knowledge of old methods and added new ones for the more certain differentiation of the various forms in which fats and fat-like substances occur in the organism. In this note no attempt is made to enumerate the many substances which will stain fat (Eisenberg); only those more recent advances are briefly detailed which have placed the staining of fatty substances on a more certain chemical basis.

Osmic acid (osmium tetroxide, OsO_4) was formerly employed as a stain for all tissue fats, but it is now generally agreed that osmic acid is only a stain for fatty compounds containing one or more unsaturated fatty acid radicals. The ordinary neutral fats of the body are mixtures of the triglycerides of palmitic, stearic, and oleic acids. Tristearin and tripalmitin are saturated compounds and do not blacken when treated with osmic acid. That is to say, they do not reduce, being already saturated, the soluble OsO_4 to the insoluble black oxide OsO_2. Triolein on the contrary, with its three unsaturated oleic acid radicals, takes up oxygen with avidity from OsO_4, causing a deposition of the oxide, OsO_2.

Osmic acid is thus seen to be unreliable as a stain for all fats. Oleic acid, on the other hand, is seldom if ever absent from the body mixtures of neutral fats, so that osmic acid is not to be discarded, but rather carefully controlled by more reliable stains, such as Sudan III. The one advantage which until recently osmic acid has possessed over other methods is that it renders the fats insoluble in the usual fat solvents, and hence capable of being sectioned in paraffin. But this supposed advantage has been greatly overestimated. It is certainly true that small droplets of fat blackened by osmic acid may be dissolved completely by alcohol, while the morphology of larger drops is seriously altered. Nor are variations in the depth of staining tone of various fats after osmium treatment reliable criteria of their composition, as has been assumed by some observers. It must also be borne in mind that tissues treated with osmic acid and subsequently with alcohol, may become blackened in some places where no fat is present. Thorough washing tends to prevent this. It would seem necessary to reduce further the OsO_2 by some such reducing agent as pyroligneous acid, as embodied in Benda's method for the demonstration of cell granules.

Sudan III and Scharlach R. are far more reliable fat stains than osmic acid. Until recently they have been applied for obvious reasons only to frozen sections, but results to be mentioned presently seem to widen greatly their usefulness by permitting their application to paraffin material. They color specifically the neutral fats, tinging slightly the surrounding tissues. Fats are not chemically altered by Sudan III or Scharlach R., and are stained owing to the fact that these dyes, in common with many others, are more soluble in fat than in alcohol. For the demonstration of neutral fats, Sudan III and Scharlach R. leave nothing to be desired, but they do not stain differentially other fatty substances, such as fatty acid, soaps, and lipoids.

The important place which the lipoid substances have been shown to occupy, both in the normal and pathological metabolism of the cell, have rendered impera-

tive the development of new methods for their morphological study. Histologists have long used Weigert's method for staining a mixture of lipoid substances, myelin, in the axis cylinder sheaths of medullated nerves. A brief discussion of this stain in the light of the recent very important work of Smith, Mair, and Thorpe (13) will render clearer some of the problems connected with the specific staining of various lipoids. The term myelin, introduced by Virchow, is not used to designate a definite chemical compound of constant composition, but rather a physical complex of various lipoids which is characterized by certain peculiar properties. Virchow (14) defined the properties of the myelin substances which he had found widely distributed throughout the body as follows:

1. They swell in water, exhibiting the most irregular, bizarre forms, changing their shape while under observation (myelin figures).

2. Easily soluble in hot alcohol; precipitated only in part by cooling.

3. Soluble in ether, chloroform, and turpentine.

4. Affected very slightly by weak acids and alkalis; shrink and lose their peculiar property of myelin figure formation in strong alkalis. Mettenheimer, in 1858, first noted that the myelin bodies when examined under crossed Nicol prisms are anisotropic. Such anisotropic myelin bodies are fluid crystals (Adami (15)). They may be brilliantly demonstrated under the microscope by adding a drop of strong ammonium hydroxid solution to a drop of oleic acid. The following substances are capable of yielding anisotropic myelin bodies (Aschoff (16)): (1) the phosphatids; (2) soaps of oleic acid; (3) esters of cholesterol; (4) solutions of cholesterol in phosphatids, fatty acids, and fats; (5) solutions of cholesterol esters in fats.

Smith, Mair, and Thorpe have carefully investigated the chemical reactions underlying Weigert's method for staining the myelin sheath of nerves, and other myelin bodies. As is well known, Weigert's method consists in thoroughly chromatizing material to be stained by prolonged exposure to potassium dichromate and then deeply staining the sections with hematoxylin. Upon properly differentiating, a lake of hematoxylin is found deposited in the myelin substance of the nerves. Smith and his co-workers have demonstrated that any fatty body containing an unsaturated grouping in its molecular constitution will stain by Weigert's method, whereas saturated fatty compounds, such as tripalmitin, do not stain thus. They have further shown that when unsaturated compounds, such as triolein, are treated with potassium dichromate, one molecule of CrO_2 attaches itself to each unsaturated acid residue converting it into an insoluble hydroxyacid and, upon subsequent treatment with hematoxylin, an insoluble lake is formed in the fat. Now the ordinary neutral fats in the body are mixtures containing triolein, tristearin, and tripalmitin, and since triolein contains unsaturated acid radicals, the neutral fats of the body should stain by Weigert's method. Such indeed is the case. The neutral fat mixtures differ, however, from the myelin of the nerve sheaths in requiring much more time for the oxidative action of potassium dichromate, like quantities of the two substances being used. So the reaction, as Smith points out, is not altogether chemical. The physical complex, myelin, oxidizes much more rapidly than the same volume of neutral fat mixture. Smith, Mair, and Thorpe thereupon investigated the staining properties of artificially prepared myelins, such as mixtures of

cholesterol and lauric acid. Neither pure cholesterol nor lauric acid stains by Weigert's method, but when mixed they yield myelin figures and stain readily by Weigert's procedure. In other words, two chemical substances which alone are non-oxidizable with potassium dichromate, are easily oxidized when united in a molecular arrangement which yields myelin figures.

Myelin bodies occur under both physiological and pathological conditions in various cells of the body (Aschoff (16)). Applying the results of the work of Smith, Mair, and Thorpe to the body cells in general, Dietrich (17) has shown that myelin substances can be stained differentially from neutral fats.

Neutral fats treated with potassium dichromate for a sufficient length of time are rendered insoluble, and may be sectioned in paraffin and subsequently stained with Sudan III or Scharlach R. In conclusion it is interesting to note that one simple chemical change is essentially the basis of the Weigert, Marchi, and osmic acid methods of staining fatty substances. This change consists in an oxidation of the unsaturated fatty acid radicals present in the molecules of certain fats and lipoids.

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EXPLANATION OF PLATE XXXVII.

FIGS. 1 and 2 are microphotographs taken under exactly the same magnification.

FIG. 1. Section of normal pig testis in active spermatogenesis. Altmann's fixation (osmic acid and potassium dichromate); stained with Bensley's neutral gentian. The droplets of neutral fat lying in the Sertoli cells form an outline of the seminal tubule. Numerous small droplets of fatty material are seen toward the lumen.

FIG. 2. Section of cryptorchid pig testis, stained with Sudan III and hematoxylin. The tubules are much smaller than in the normal testicle, and are lined only by a layer of Sertoli cells, which contain a large number of neutral fat droplets. No sperm-forming cells are present. The Leydig cells are seen, on comparison with Fig. 1, to be greatly increased in number.

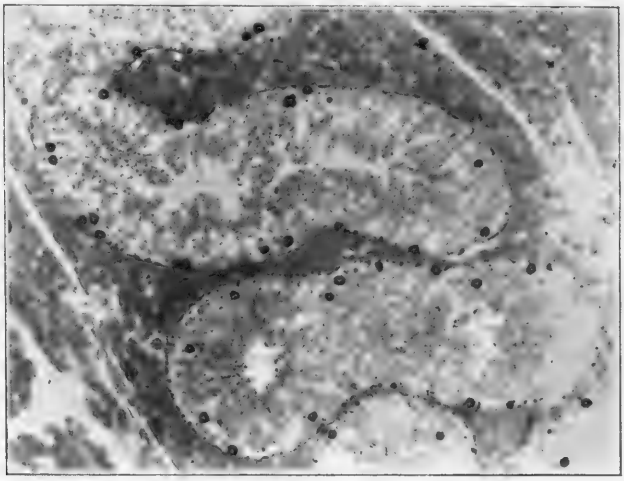

FIG. 1.

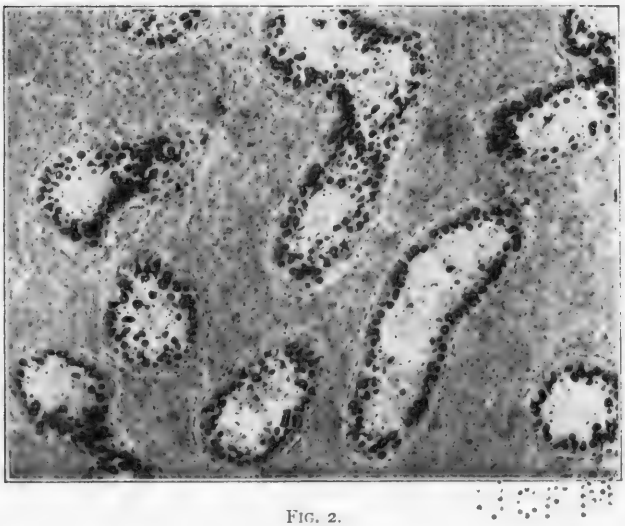

FIG. 2.

THE CULTIVATION OF THE LEPROSY BACILLUS FROM THE HUMAN TISSUES WITH SPECIAL REFERENCE TO THE AMINO-ACIDS AS CULTURE MEDIA.*

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In a previous communication,[1] I pointed out that although the leprosy bacillus will readily multiply in pure culture upon a variety of media, after it has once acquired more or less saprophytic properties the initial multiplication outside of the animal body is effected, on the contrary, with extraordinary difficulty and not at all unless special media and methods are employed. This paper aims to give briefly the results of my experience with the amino-acids in the cultivation of the lepra bacillus and to discuss the most advantageous methods of its isolation. There are several ways in which the organism may be cultivated from the tissues, but two in particular are worthy of special consideration and will be described in detail.

When special food stuffs are seeded simultaneously with some bacterial species which are known to aid the artificial growth of *Bacillus lepræ*, the latter increase in number very slowly and do not show macroscopic growth for several weeks, while generations removed from this parent stem multiply rapidly and reach under the same conditions a maximum growth in two to three days.

This wide difference in the cultural behavior of lepra bacilli under conditions of artificial environment is not unusual since other pathogenic microörganisms behave in a similar manner. In this respect *Bacillus lepræ* has its prototype in certain strains of the tubercle family which at first grow feebly, and in some instances not at all, on the most favorable artificial medium. The fact that the initial growth of the leprosy bacillus is so difficult to obtain

* Received for publication, January 13, 1911.

[1] *Jour. Exper. Med.*, 1910, xii, 649.

even in the presence of special foodstuffs, although once cultivated it grows profusely on ordinary media, is not surprising when we consider that *Bacillus tuberculosis* is incapable of cultivation directly from the tissues on glycerine agar, upon which medium it grows well in subsequent generations.

Rapid growth of *Bacillus lepræ* indicates, of course, frequent division of the bacterial cell and is a property quickly acquired by cultures that are no longer influenced by the conditions of the host. In the animal tissues, division of the leprosy organisms takes place slowly, and probably more slowly than that of the tubercle bacillus under similar conditions. In this connection it is noteworthy that the bacilli in the first generations on artificial media, like those in the tissues, multiply very slowly and are long, slender, and distinctly "beaded," while those several generations removed from the parent stem grow with great rapidity and are short, almost coccoid, and devoid of "beads." Since "beading" of the bacilli always occurs in the animal body and persists for a short time only in artificial cultures, it is not only possible but highly probable that the leprosy bacillus after all is not a true parasite in the strict sense of the term. The difficulty with which animals are infected, including the human subject, and the commensal relationship enjoyed by the bacilli in the tissues of man strengthens such an hypothesis. The transformation in morphology of the organism has been fully described in a recent paper.[2]

Clegg[3] was the first to show that the acid-fast bacilli in leprous tissue will multiply upon an artificial medium and continue to grow in subculture. His results were obtained with ameba and their symbiotic bacteria from which pure cultures of *Bacillus lepræ* were subsequently derived by heating at 60° C. for thirty minutes, which temperature destroyed the ameba and symbiotic bacteria without injuring the acid-fast organisms.

Soon after the publication of Clegg's paper, the author confirmed his results and, moreover, succeeded in the isolation and cultivation of *Bacillus lepræ* directly from the human tissue without the aid of ameba or bacteria upon a tryptophane medium. At this time

[2] *New Orleans Med. and Surg. Jour.*, 1911, lxiii, 549.
[3] *Philippine Jour. Sc.*, 1909, iv, 403.

it was noted that the specific organism of leprosy would multiply in and around the transplanted pieces of leprous tissue upon an ordinary blood agar medium which had been seeded with influenza bacilli or with meningococci. No attempt was then made to explain the occurrence of the growth of leprosy bacilli in association with other bacteria aside from the general notion that the transferred tissue was responsible. While the tissues do play a part, recent work tends to show that the associated bacteria play by far the more important part.

It can be definitely stated that the initial growth of *Bacillus lepræ* upon a medium of ameba and bacterial symbiotics is the result of the reaction of the associated bacteria; namely, *Bacillus typhosus, dysenteriæ, coli, cholera,* etc. This is contrary to the view first formed by me in a previous paper[4] in which I stated that the ameba in some way supplied the necessary nutriment for the lepra bacilli; recent tests, however, do not support this contention.

Experiments show that amebæ are in no way concerned in the multiplication of *Bacillus lepræ* and should not be employed in the culture medium. Growth of leprosy bacilli is not aided in the least by either the encysted or ameboid forms; but on the contrary the vegetative type of the protozoan may prove a serious hindrance to the initial growth of the bacilli, since they thrive better in symbiosis with pure cultures of leprosy than they do with any of the typhoid-colon group. Encystation does not occur as early with cultures of *Bacillus lepræ* and this may be attributed to the fact that leprosy bacilli live for months and are constantly multiplying, whereas the typhoid colon group of bacilli live for weeks only, and ameba appear not to feed upon dead cultures.

The ordinary laboratory media may be successfully employed to recover *Bacillus lepræ* if bits of tissue are carried over to the culture medium and bacilli such as the colon, typhoid, proteus, subtilis, etc., are added at the same time. If the tissue is already contaminated by bacteria there is no necessity for this addition, as the contaminators answer the purpose equally well.

The primary multiplication of *Bacillus lepræ* in excised bits of tis-

[4] *Jour. Exper. Med., loc cit.*

sues in the presence of the common bacterial species is caused by the chemical action of the extraneous bacteria on the cells of the tissue whereby the nucleo-proteids are split up and changed. These final products of tryptic digestion, of which tryptophane is one, are capable of being assimilated by the leprosy bacilli through which their growth is promoted. *Bacillus lepræ*, it would seem, is unable to use the albumen molecule as such, but can readily assimilate the split products. This explains why growth of the bacilli occurs when extraneous bacteria are present, and the failure to multiply where they are absent, unless some of the cleavage products are included as part of the artificial medium. In support of this explanation repeated tests have shown that growth will not occur where the lepra bacilli are first washed free from tissue cells before seeding with the other bacteria. On the other hand, the acid-fast bacilli in excised bits of leprous tissue that are contaminated with putrefactive bacteria, steadily increase in number when kept even at room temperature in distilled water or salt solution; while in uncontaminated leprous tissue under the same conditions the lepra bacilli will not multiply though they remain viable for months, as shown by subsequent cultural or animal tests.

It is well known that putrefactive bacteria and many of the intestinal species will cause disintegration of the albumen molecule with the formation of albumoses, peptone, and finally amino-acids. In other words, certain bacterial species behave towards the nucleo-proteids in the same way as trypsin. Apparently *Bacillus lepræ* itself has no power to split or hydrolize the albumen moiety.

Obviously then two methods may be followed in the initial cultivation of *Bacillus lepræ* from the leprous tissue, (1) the use of a special medium containing amino-acids (direct method), and (2) the addition of some bacterium to the culture medium which is capable of hydrolizing the transferred bits of infected tissues (indirect or bacterial method).

The direct method has the advantage of simplicity and requires no subsequent plating or heating to obtain a pure culture and is applicable in all cases where pieces of tissues can not be obtained. On the other hand, though the indirect method requires no special

bacteriological technique to provide against contamination, it does necessitate subsequent replating or heating of the growth in order to separate *Bacillus leprœ* from the associated bacteria. Besides, it is also necessary to transfer the pure colonies to an amino-acid medium to insure continued growth until the culture is accustomed to the new environment. Although both methods have their advantages and disadvantages, the former is to be preferred for practical purposes.

METHOD OF CULTIVATION.

In a previous communication,[5] the use of tryptophane and other amino-acids was recommended for the direct isolation of *Bacillus leprœ* from the tissues, but as these substances are difficult to obtain and prepare I have recently used a medium composed of albumen and trypsin which gives equally good results. In this medium the necessary amount of digestion of the nucleo-proteid is secured by the trypsin. Its preparation and method of use follow: egg albumen or human blood serum is poured into sterile Petri dishes and inspissated for three hours at 70° C. The excised leprous nodule is then cut into thin slices, two to four millimeters in breadth and five-tenths to one millimeter in thickness, which are distributed over the surface of the coagulated albumen. By means of a pipette the medium thus seeded with bits of tissue is bathed in a 1 per cent. sterile solution of trypsin, care being taken not to submerge the pieces of leprous tissue. Sufficient fluid is added to moisten thoroughly the surface of the medium.

The Petri plates are now placed in a moist chamber at 37° C. and allowed to incubate for a week to ten days. They are removed from the plates from time to time, as evaporation necessitates, for the addition of more trypsin. It will be noted that after a week or ten days the tissue bits are partially sunken below the surface of the medium and are softened to a thick, creamy consistence, fragments of which are readily removed with the platinum needle. On microscopic examination of this material it is noted that the leprosy bacilli have increased to enormous numbers and scarcely a trace of the tissues remain. Separate lepra bacilli colonies are also discernible on and around the softened tissue masses, and those

[5] *Jour. Exper. Med., loc. cit.*

furthest away from the masses attain a size of two millimeters in diameter. The colonies at first are greyish white, but after several days they assume a distinct orange yellow tint.

From the plates subcultures may be obtained by transferring portions of the growth to a second series of plates or to slanted culture tubes that contain the special albumen trypsin medium. After the third or fourth generation, the bacilli may be grown without difficulty upon glycerinated serum-agar prepared in the following manner: twenty grams of agar, three grams of sodium chloride, thirty cubic centimeters of glycerin, and 500 cubic centimeters of distilled water are thoroughly mixed, clarified, and sterilized in the usual way. To tubes containing ten cubic centimeters of this material is added in proper proportion a solution of unheated turtle muscle infusion. Five hundred grams of turtle muscle are cut into fine pieces and placed in a flask with 500 cubic centimeters of distilled water. This is kept in the ice chest for forty-eight hours and then filtered through gauze to remove the tissue. The filtrate is then passed through a Berkefeld filter for purposes of sterilization. By means of a sterile pipette, five cubic centimeters of the muscle filtrate is added to the agar mixture which has been melted and cooled at 42° C. The tubes now are thoroughly agitated and allowed to solidify in the slanted position.

This medium is perfectly clear, or light amber in color, and admirably suited for the cultivation of *Bacillus lepræ* once the initial culture has been started. Growth is luxuriant and reaches its maximum in forty-eight to sixty-four hours. On the surface of this medium the growth is moist and orange yellow in color, while in the water of condensation, though growth apparently has not occurred, the detached bacilli collect in the dependent parts in the form of feathery masses without clouding the fluid.

Ordinary nutrient agar may be used with trypsin as a plating medium instead of the inspissated serum where tissue bits are employed. With the addition of 1 per cent. of tryptophane it answers every purpose, whether the bacilli are planted with tissues or alone. It also serves to start multiplication of lepra bacilli that are contaminated at the time of plating. In the latter case the medium is "surface seeded" with an emulsion of the tissue juices in the same

manner as in preparing "streak" plates. The leprosy colonies in the thinner parts of the loop track are well separated and easily distinguished from those of the other species by their color and by their appearance only after two to five days.

In using the indirect method for the cultivation of *Bacillus lepræ* it is necessary only to provide the pieces of infected tissue with some bacterium capable of oxidizing the nucleo-proteids. A great variety of bacteria may be utilized for this purpose; among them may be mentioned *B. typhosus, paratyphosus, prodigiosus, pyocyaneus, dysenteriæ,* etc. In using the indirect method it is best not to employ a spore-bearing species as they are much harder to get rid of subsequently, though many of them answer the purpose equally well. Not infrequently a spore-bearing variety will be encountered in the tissues at the time of removal, but this is of little consequence as a second plating readily separates the contaminator. Putrefactive bacteria apparently do not interfere in the slightest degree with the multiplication of *Bacillus lepræ,* and when they hydrolize the tissue they serve as a valuable adjunct by converting the nucleo-proteid into suitable nutritives.

In using an agar medium it is well to leave out peptone and to titrate the reaction to 1.5 per cent. alkaline in order to prevent a too profuse growth of the associated bacteria; besides, an alkaline medium seems best adopted for the multiplication of the lepra bacilli. The method is to be recommended in all cases where the leprous tissue is contaminated with other microörganisms, as, for example, material from the naso-pharyngeal lesions or those on the exposed surfaces of the body.

As in the direct method, small bits of leprous tissue are placed upon the surface of the solidified alkaline agar and moistened with a thin suspension of some one of the bacterial species above mentioned. The plates are now incubated at 32° to 35° C. for two weeks, when they may be removed and portions of the softened tissue masses streaked over the surface of a second series of plates in order to obtain separated lepra bacilli colonies. It is necessary to use for the second plating either the trypsin-albumen medium or tryptophane agar.

Nutrient agar or any of the ordinary laboratory media will not serve as a transfer medium until the cultures have become accustomed to artificial conditions. So if the trypsin-albumen or tryptophane is not used it is necessary to keep the cultures growing with the associated bacteria for a much longer time, *i. e.,* until the lepra bacilli have acquired saprophytic properties. Recently isolated cultures require the dissociate products of tryptic digestion whether they have been previously produced in the medium or converted *in situ* by certain other bacteria. Glycerine added to the medium seems to enhance its nutritive value; at least the growth that results in its presence is more luxuriant.

Bacillus lepræ will also grow on the various blood agar media once they are accustomed to artificial conditions. The Novy-Mc-Neal agar for the cultivation of trypanosomes gives a luxuriant growth of the organism if 2 per cent. glycerine has been added; without the glycerine, growth is very scant. Fluid media are not suited for the artificial cultivation of leprosy bacilli unless they are kept upon the surface. Like the tubercle bacillus they require abundant oxygen; those sinking beneath the surface cease to multiply but remain viable for months. Where it is desired to study their fermentative properties it is necessary first to grow the lepra organism on a solid medium and later to transfer parts of the growth to sterile floats (paper) that are carefully placed upon the surface of the fluid.

Ordinarily the growth of *Bacillus lepræ* is very moist and in this respect unlike that of *Bacillus tuberculosis,* except possibly the avian strain. Sometimes when the medium is devoid of water of condensation the growth is dry and occasionally wrinkled, though it is easily removed from the surface of the medium.

The chromogenic property of lepra cultures is a constant and characteristic feature for the rapidly growing stains. The color varies in degree of intensity depending upon the medium employed. If glycerinated agar (without peptone) is used, the colonies are faint lemon yellow, while on inspissated blood serum they are deep orange. It is noteworthy that the growth in the tissues and in the first dozen or so generations on artificial media is entirely without pigment.

CONCLUSIONS.

In the cultivation of *Bacillus lepræ* the initial multiplication outside the body cannot be obtained unless amino-acids are present in the medium. The amino-acids are believed to be essential nutritives for the initial growth of the organisms.

It has been demonstrated that the primary growth of the leprosy bacilli occurs only in the presence of the products of tryptic digestion.

Hence, putrefactive and other bacteria which are capable of splitting nucleo-proteids into their end acid products are, in consequence, of value in the isolation and cultivation of the leprosy bacilli. Amebæ are not necessary for securing the primary multiplication of the leprosy bacilli upon artificial media and are detrimental since they feed with avidity upon the bacilli themselves.

Two methods may be employed for recovering in culture *Bacillus lepræ* from the tissues. In one (the direct), tryptophane or a mixture of albumen and trypsin are employed with a culture medium; in the other (indirect), bacterial species capable of digesting the albumen constituent of the culture medium are introduced into the medium. In both, the end result is identical, since they both provide for the presence of the amino-acids in the medium, without which the primary multiplication of the leprosy bacilli cannot be secured.

THE EXPERIMENTAL PRODUCTION OF LEPROSY IN THE MONKEY (MACACUS RHESUS).*

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Many investigators have attempted to reproduce leprosy of man in the lower animals under natural and various artificial conditions, but aside from a slight local reaction that has occasionally developed at the site of inoculation the reported results have been invariably negative. Certainly it cannot be claimed that the type of the disease as we see it in man has been produced under any conditions in the lower animals. Recently Clegg,[1] Sugai,[2] and the author,[3] among others, have shown that leprous lesions occur in the guinea pig and Japanese and white mice following intraperitoneal or subcutaneous injections of cultures of leprosy bacilli or the infected human tissues, but in no instance has the clinical picture of human leprosy manifested itself in these animals.

Nicolle[4] claims to have produced by subcutaneous inoculation of bits of leprous tissue typical leprous nodules in the monkey. However, he does not state that in these animals there developed leprosy with its symptom-complex as we see it in man.

The failure to occasion more than a localized infection in the experimental animal with emulsions of lepra bacilli obtained from bits of human tissue may be attributed in part, possibly, to the fact that in the tissues the bacilli are too few in number and for the most part are non-viable. It sometimes happens in experimental tuberculosis that we fail to infect the susceptible guinea pig with human tuberculous material, and this is explained by the scarcity

* Received for publication, January 13, 1910.

[1] *Philippine Jour. Sci.*, 1909, iv, 403.

[2] *Lepra*, 1909, viii, 203.

[3] *Jour. Exper. Med.*, 1910, xii, 649; *Arch. Int. Med.*, 1911, vii (in press).

[4] *Semaine méd.*, 1905, xxv, 116.

of bacilli in the tissue employed for inoculation and by the circumstances that many of them are avirulent or dead. Failure is even more to be expected with experimental leprosy since the animals used are certainly less susceptible to leprosy than to tuberculosis.

Prior to the cultivation of *Bacillus lepræ* outside of the animal body, it was only possible to work with the infected human tissue; and judging from the small number of bacilli that multiply when this material is transferred to a suitable artificial culture medium, it may be asserted that in leprosy more than in tuberculosis relatively fewer organisms are living; in my opinion this explains the failure of former workers to transmit leprosy to the monkey by direct implantation of human leprous tissue. On the other hand, when pure cultures are available and large quantities of viable leprosy bacilli are injected at given intervals, typical leprosy of the tubercular type may be reproduced in the *Macacus rhesus* monkey.

In this paper I wish to give briefly the results of some experiments upon monkeys in which disseminated leprosy has followed the repeated injection of large quantities of leprosy cultures. The cultures used were grown on slanted glycerinated blood agar for forty-eight hours and the growth washed down in five cubic centimeters of normal saline solution and thoroughly emulsified, and the suspension so prepared was employed as the injection material. Three strains of *Bacillus lepræ* were utilized in the experiments, one of which has been under cultivation for about a year and is now more than twenty generations removed from the parent stem, and the others have been growing upon artificial media six and nine months respectively.

EXPERIMENTAL PART.

Experiment I.—On October 17, 1910, a full-grown monkey (*Macacus rhesus*) was inoculated hypodermatically into the subcutaneous tissues of the right inguinal region with one cubic centimeter of a heavy suspension of leprosy bacilli. Twenty-four hours after the injection the monkey appeared perfectly well and showed no signs of inflammation at the site of inoculation. Eight days later (October 25) the animal was given a second injection of the

culture into the subcutaneous tissues of the right breast. At this time there was still no evidence of either localized or general infection resulting from the first injection. The monkey was apparently in perfect health. One week after the second inoculation, or eighteen days after the first, a nodule the size of a split pea developed in the subcutis over the right groin at the site of the first inoculation, which slowly increased in size from day to day and in three weeks attained the size of a pigeon's egg. At this time a small nodule also appeared at the site of the second inoculation. On November 1 the animal received a third injection of the culture into the subcutaneous tissue over the right groin which was followed in forty-eight hours by the appearance of a hard nodular mass that has slowly continued to increase in size. The skin about this nodule showed numerous smaller indurated areas. The animal received a fourth injection of the culture into the subcutaneous tissue of the left breast on November 16. In three days a purplish red tumor approximately two centimeters in diameter developed and two weeks later the overlying skin became firmly adherent, thin, and shiny. This tumor mass ruptured on the seventeenth day discharging a grumous material which on microscopic examination contained large numbers of leproid cells filled with acid-fast bacilli. Cultures prepared from this material remained sterile with respect to pyogenic microörganisms; but *Bacillus leprœ* was recovered in pure culture upon special artificial media.

One of the nodules in the groin that seemed to fluctuate on pressure and was similar in other respects to the nodule in the breast that opened spontaneously, was incised and found to consist of a homogeneous, semigelatinous material showing no evidence of tissue necrosis. In color and consistence the cut surface of the mass resembled an incised lymph gland. Surrounding the nodule was a narrow hemorrhagic zone which was sharply defined and did not extend into the tumor mass.

Forty-six days after the first injection the monkey showed typical signs of disseminated infection and presented the clinical picture of human leprosy of the tubercular type of the disease. On the right side of the face a small raised erythematous patch approximately

three centimeters in extent appeared and gradually extended over the bridge of the nose, right eye, and forehead. The appearance of the patch was identical with the erythema noted in human leprosy during certain periods of the disease. This area was incised and the expressed bloody serum examined and numbers of acid-fast bacilli were found. Five days later, although the redness of the cheek and forehead had faded, the skin was left somewhat thickened and distinctly pigmented and was, therefore, in striking contrast with the uninvolved part of the face. The skin over the superciliary ridges also became reddish pink and roughened; while between the eye-brows there appeared several small elevated hard nodular masses.

The large nodules in the groin and chest have lost all sensation to pain as determined by the fact that the animal permits free incision without flinching, but evinces unmistakable signs of pain as soon as the knife extends into the surrounding normal skin. About the nodules, for a radius of two to three centimeters, the skin is distinctly hypersensitive, the slightest touch causing the animal to flinch.

Experiment II.—On November 4, a female *Macacus rhesus* monkey was given subcutaneously into the chest one cubic centimeter of an emulsion of the necrotic material obtained from one of the nodules of monkey 1. This material contained a great number of bacilli, but judging from the amount of growth that resulted in isolated patches over a thickly seeded artificial medium only a few of the bacilli were viable or capable of multiplication. Two weeks after the injection a small hard flattened nodule could be palpated which gradually subsided and in ten days to two weeks had completely disappeared. At this time the monkey was given a second injection subcutaneously into the left groin using the entire growth from a four day old slant culture. Five days later the animal showed at the site of the second inoculation a purplish red nodule approximately one centimeter in diameter, which at present is steadily increasing in size. Already there are analgesia and hypersensitiveness associated with the lesion. There is, however, no evidence as yet of superficial lesions elsewhere on the body. The animal received a third injection of the culture on November 18 into

the loose subcutaneous tissues of the right vulva. Forty-eight hours after the inoculation the vulva was swollen to three times its normal size and presented a deep purplish red color. On November 24 a macular eruption appeared on the chest in the mid-sternal region which persisted for three to four days when it disappeared, leaving the skin roughened and of a dusky gray discoloration. As in the first monkey, acid-fast bacilli were demonstrated in the bloody serum obtained from the incised macular lesion.

In the attempt to infect by way of the circulation, a third monkey, an adult male, was given intravenously into the left femoral vein a heavy suspension of leprosy bacilli. The animal received in the same manner a second injection one week after the first, using twice the amount of culture previously employed. At the present writing there is no evidence of cutaneous lesions or signs of infection elsewhere in the body. However, it is too early to expect skin lesions, judging from the time it requires to produce them in monkeys that have received subcutaneous injections.

The skin lesions are primarily exudative in character, and the exudation is soon followed by proliferation of the fixed cells in the area involved. The sections from the excised nodules experimentally produced are indistinguishable microscopically from those of human cases. The earliest observed changes in the tissues consist of a collection of lymphoid and plasma cells, the latter predominating, while the older or more advanced nodules showed great numbers of the so-called lepra and epithelioid cells intermingled in a loose matrix of young connective tissue. Where the nodule develops rapidly the central part sometimes undergoes a form of liquefaction necrosis. Associated with the lesion is always a marked dilatation of the bordering capillaries which gives to the overlying skin its dark purplish red color. The smaller vessels in the immediate neighborhood show a perivascular infiltration with lymphoid and plasma cells; while the nerves in and about the nodules are often more or less completely surrounded and pressed upon by proliferated cells of epithelioid type. The nerve fibers in the older nodules show evidence of degeneration which apparently is the result of pressure of the surrounding cells rather than of invasion of the nerve sheaths by the bacilli.

The lepra bacilli are chiefly contained within cells, very few being extra-cellular. Even in specimens obtained from the center of broken down nodules extra-cellular organisms are only exceptionally found.

Whether repeated inoculations of large numbers of viable lepra bacilli are necessary to cause generalized infection in the monkey is a question as yet undetermined. Considering the fact that the monkey is not highly susceptible to the disease under ordinary conditions, it is reasonable to assume that until the bacilli become parasitic in the new host only a few of them will survive in the first transfers. However, those that live and multiply in the monkey may in the course of time become permanently parasitic so that subsequent passages through other individuals of the species may be followed by more profound results.

The production of leprosy in the monkey is of far greater importance than the successful inoculation of smaller animals, as it offers a better opportunity to study the experimental disease and to work out problems that have a bearing on the pathology of the human disease. The successful transmission of clinical leprosy to the monkey establishes beyond doubt the leprous nature of the acid-fast bacilli cultivated upon artificial media from human leprous tissue, and proves conclusively that some strains, at least, retain their infectivity under conditions of artificial growth for more than a year. The experiments also bring out the necessity of first cultivating the bacilli on an artificial medium in order to obtain large numbers of viable organisms with which to accomplish the experimental production of the disease.

The analgesia of the leprous nodules with which are associated hypersensitive zones in the monkey happened to be a marked feature of the cutaneous lesions of leprosy in man. This fact and that of the occurrence of erythematous patches, thickening and pigmentation of the skin in areas far removed from the inoculated site in the monkey, seem to prove conclusively the spread of the infection from one side to another, and to justify the statement that leprosy is reproducible in the monkey, in which animal many of the clinical and pathological phenomena common also to the human subject may be advantageously studied.

CHEMO-IMMUNOLOGICAL STUDIES ON LOCALIZED INFECTIONS.

Second Paper: Lysis of the Pneumococcus and Hemolysis by Certain Fatty Acids and Their Alkali Soaps.*

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In a former paper[1] the action on the pneumococcus of sodium oleate alone and in combination with an immune antipneumococcus serum was described and the results of the application of this action to experimental pneumococcus infections were given. The present paper deals with the extension of the study of lysis of the pneumococcus, in which the comparative activities of certain other unsaturated fatty acids and soaps have been tested. In view of the close relationship which early manifested itself between the lytic power of these compounds for the pneumococcus and for red blood cells the study was made to include hemolysis also. This parallelism between lysis of red blood cells and of virulent pneumococci, which exists also in the action of bile, is all the more striking when compared with the action of soaps upon other bacteria. Streptococcus, for instance, is readily killed by oleic acid soaps but, in contra-distinction to pneumococcus, it is not dissolved.

The fatty acids which have been tested comprised a mixture of unsaturated acids obtained from the livers of rabbits poisoned with phosphorus, and also several single isolated acids of known chemical composition and structure. The former will be considered first.

FATTY ACIDS IN THE LIVER IN EXPERIMENTAL PHOSPHORUS POISONING.

The liver in experimental phosphorus poisoning was chosen as a source of unsaturated fatty acids because it has been shown to

* Received for publication, January 14, 1911.

[1] *Jour. Exper. Med.*, 1911, xiii, 1.

contain them in relatively large quantities, and also because of the difficulty of obtaining unsaturated acids from the trade.

The difference in content of fatty acids between the normal liver and the liver in phosphorus poisoning is partly a quantitative one but especially one of state.

Hartley[2] showed that approximately one-half of the higher fatty acids in the normal liver of the pig are unsaturated acids existing to a considerable extent in lecithin and similar complexes. He proved the presence of oleic and of linoleic acids and obtained evidence which pointed to the presence of an acid having the formula $C_{20}H_{32}O_2$ and forming about 10 per cent. of the total fatty acids. Mansfeld[3] recently found that the normal liver upon extraction with ether yielded as free fat only one-half or less of its total fat, the remainder being bound with protein in such a way as to be insusceptible of being extracted with ether unless the protein was first digested or denaturized. In marked contrast, the liver in phosphorus poisoning yielded all of its fat, from which he concluded that not only the fat which had been deposited in the liver but also that which existed there already was free. He noted further that in advanced degeneration caused by phosphorus some of the fatty compounds were split into free acid and glycerin. Similarly, Joannovics and Pick[4] observed in poisoning by toluylendiamin and phosphorus and in acute yellow atrophy that powerfully hemolytic unsaturated acids existed free in the liver in much greater quantity than in the normal liver; and confirmatory observations to these have been recorded by Ehrmann and Stern[5] and by Jacoby.[6]

We employed a number of rabbits to which phosphorus was given sometimes in the form of pills by mouth, but usually as the oil injected subcutaneously. After bits of tissue were taken for sections the livers were finely ground, mixed, and weighed, and the moist material extracted in the manner to be described later.

In order to secure information concerning the nature and degree of fatty change and the relation between neutral fats and phosphatides, a duplicate series of sections was prepared, the one cut from frozen tissue and stained with Sudan III, the other mordanted with potassium bichromate, imbedded in paraffin, and then stained with Sudan III, according to the method of Ciaccio for lecithin. When sections are prepared in the latter way neutral

[2] *Jour. Physiol.*, 1907, xxxvi, 17; 1909, xxxviii, 353.

[3] *Arch. f. Physiol.*, 1909, cxxix, 46.

[4] *Ztschr. f. exper. Path. u. Therap.*, 1909, vii, 185; *Berl. klin. Wchnschr.*, 1910, xlvii, 928.

[5] *Berl. klin. Wchnschr.*, 1910, xlvii, 282.

[6] *Idem*, 1910, xlvii, 677.

fats, free fatty acids, and cholesterin are dissolved away while the lecithins remain to take up the stain. With the help of this method Ciaccio[7] has made a contribution to the study of lipoids in stained sections similar to that made by Kaiserling and Orgler, who first awakened interest in the polariscopic differentiation of certain lipoids from neutral fats. He has supplied evidence which points to a special lecithin metabolism in hematopoietic tissue and also to a peculiar lecithin degeneration in distinction to fatty degeneration.

A comparative study of the sections prepared as stated showed that in all of the livers, most strikingly in the extremely fatty ones, about one-half of the fat consisted of phosphatides, which took on an orange yellow color in the imbedded tissue, while all of the fat in the frozen sections was stained a deep red. Both the neutral fats and phosphatides in some of the livers appeared as extremely fine granules, resolved only by the high power, studding the cells; in others they occurred in small and large drops completely filling the cytoplasm of the cells.

EXTRACTION OF THE ORGANS.

1,260 grams of the fresh, finely ground liver substance were extracted in portions with about three volumes of absolute alcohol at 36° C. for three or four days, the alcohol being renewed twice. After decantation of the last alcohol, the pulp was expressed in a fine cloth, dried at 36° C., and then extracted for two or three days with Merck's dry reagent ether in the Soxhlet apparatus. The combined alcoholic extracts were evaporated in the fan drying apparatus and the residue extracted with several portions of absolute alcohol and then with ether. The last obtained alcoholic extract was dried and the residue extracted with ether. All of the ether extracts were filtered, combined, and precipitated twice with acetone. In this way, 32.44 grams of phosphatides (2.57 per cent. of the weight of the original moist material) were obtained and set aside. The remaining acetone-ether filtrate, which contained principally neutral fats, free fatty acids, and some cholesterin, was evaporated and 56.3 grams (4.47 per cent.) of residue obtained. This fraction consisted of a bright yellow material having a pungent, but not rancid, odor. At room temperature it was a thick, oily liquid with many white flakes and small masses. In the ice-chest it solidified to a yellowish white mass. A part of this fraction was extracted with several portions of absolute methyl alcohol in an attempt to separate largely the free unsaturated fatty acids as the most active hemolytic

[7] *Centralbl. f. allg. Path. u. path. Anat.*, 1909, xx, 385, 771; *Folia Haematolog.*, 1909, vii, 321; 1909, viii, 135.

constituents. The results of hemolytic tests with this methyl alcohol-soluble fraction both before and after saponification were encouraging but not satisfying. Further appropriate examination of the fraction revealed the presence in not inconsiderable quantities of cholesterin and also of solid saturated acids having a melting point at 55° C., corresponding to a mixture of stearic and palmitic acids. So that to this admixture with inactive saturated acids and inhibitory cholesterin the unsatisfactory result of the hemolytic tests was attributed.

Therefore in order more nearly completely to isolate the unsaturated acids resort was had to the lead soap-ether method. The remainder of the methyl alcohol-soluble fraction was saponified in the customary way with alcoholic caustic potash, the resulting soap solution diluted with water, neutralized with hydrochloric acid, and extracted repeatedly with ether in a separatory funnel to remove cholesterin. The soap solution was then precipitated with hot lead acetate solution, the lead soaps washed three times with hot water in the usual way, and then extracted with ether. The ether solution of lead soaps was decomposed with hydrochloric acid, the liberated fatty acids washed free of mineral acid, and the ether driven off on the water bath. The iodine value of this mixture of unsaturated acids as determined at once was 127.

Having thus secured the unsaturated acids as nearly free as practicable from inactive and inhibitory substances, we were prepared to test their action upon the pneumococcus and upon red blood cells, and to compare it with that of a few other acids.

LYTIC ACTION OF ACIDS AND SOAPS.

The available acids to be tested comprised the above described mixture of acids obtained from the phosphorus livers and composed largely of oleic and linoleic acids with probably a small quantity of still more unsaturated acids; crotonic acid, oleic acid, and erucic acid, all of the oleic series; linoleic acid, a straight chain acid of the linoleic series; and chaulmoogric acid, isomeric with linoleic but containing a closed carbon ring, and hence belonging to the cyclic series. These acids, except the last named, were Kahlbaum preparations. The soaps were made by neutralizing the acids with alcoholic caustic potash. The chaulmoogric acid was isolated from chaulmoogra oil according to the method employed by Power and Gornall,[8] who first noted its existence and described its structure. After several crystallizations from petroleum ether, the acid which we finally employed consisted of delicate white silky crystals melting at 68° and having an iodine value of 90.13, the theoretical value being 90.7.

In the hemolytic tests progressive dilutions of percentage solu-

[8] *Jour. Chem. Soc.*, 1904, lxxxv, 838.

tions, or suspensions, were employed in order to obtain comparative results of quantitative activity.

With the exception of crotonic acid which is soluble in water, the various acids were made into 10 per cent. solutions in reagent methyl alcohol and from these solutions fresh suspensions were made in distilled water,—1 per cent., 0.1 per cent., and 0.025 per cent.,—and added to the suspension of blood cells within a few seconds. Working rapidly in this way it was possible to obtain and employ fine, nearly uniform suspensions of the insoluble acids. The soaps being freely soluble in water were added in aqueous solution. Each tube contained two cubic centimeters of a 5 per cent. suspension of dog's blood cells, the requisite quantity of acid suspension or soap solution to make an ultimate dilution varying from 1–1000 to 1–120,000, and sufficient 0.9 per cent. sodium chloride solution to make the total volume three cubic centimeters. The mixtures were put into the incubator for one hour, during which time they were frequently shaken, and then removed to the ice-chest for twenty hours. It is noteworthy that the action of these substances continues in the cold.

Besides merely comparing the activity of the free acids with that of their soaps, and of the various acids and soaps among themselves, we were interested in the relation which certain characters of the compounds might bear to their lytic activity; notably, iodine value, configuration, position in homologous series, and solubility in water.

Below is given a table which embodies these points and the results of the tests, that dilution at which hemolysis was complete at the end of twenty hours being given.

A study of the table shows:

1. That the higher acids of the oleic series are very much more strongly hemolytic than the low crotonic acid. Indeed the action of crotonic acid is probably due wholly to its acidity, and the resulting hemolysis is of a different nature from that caused by the higher lipoidal acids and hence they are not strictly comparable.

2. The soaps of the higher acids are all more active than the free acids. Witness their greater solubility.

TABLE I.

Substance tested.	Formula.	Series.	Iodine value.	Solubility in water.	Complete hemolysis.
Crotonic acid.	$C_4H_6O_2$	Oleic.	295.28	12 parts.	1–5,000*
Normal potassium crotonate.				Deliquescent.	No hemolysis at 1–1,000
Oleic acid.	$C_{18}H_{34}O_2$	Oleic.	90.07	Insoluble.	1–20,000
Sodium oleate.				10 parts.	1–30,000.
Potassium oleate.				4 parts.	1–24,000
Erucic acid.	$C_{22}H_{42}O_2$	Oleic.	75.15	Insoiuble.	1–15,000
Potassium erucicate.				Slightly soluble.	1–24,000
Mixture of unsaturated acids from livers.		Oleic, linoleic, and probably $C_nH_{2n-8}O_2$.	127.3	Insoluble.	1–24,000
Potassium soaps of same				Very soluble.	50,000
Linoleic acid.	$C_{18}H_{32}O_2$	Linoleic.	181.42	Insoluble.	1–30,000
Potassium linoleate.				Very soluble.	1–100,000
Chaulmoogric acid.	$C_{18}H_{32}O_2$	Linoleic; cyclic.	90.13	Insoluble.	1–5,000
Normal potassium chaulmoograte.				Very soluble.	1–10,000
Linolenic acid	$C_{18}H_{30}O_2$	Linolenic	243.2 theoretical 274.1	Insoluble.	1–40,000
Potassium linolenate				Very soluble.	1–150,000

*Hemolysis due to acid reaction; hemoglobin discolored.

3. There is in general, among the higher acids, a close relationship between iodine value and hemolytic strength, the higher the one, the greater the other.

4. Configuration bears a relationship to hemolytic activity, since chaulmoogric acid, isomeric with linoleic, though taking up only two halogen atoms, is less active even than oleic acid.

5. No one of these characters alone serves as a reliable criterion of hemolytic power.

6. The greatest activity is shown by potassium linolenate, which exhibits a happy combination of a large number of carbon atoms, straight chain structure, high iodine value, and free solubility.

The results with oleic acid and sodium oleate are the same as those secured by Noguchi.[9] And those obtained with crotonic, oleic, and erucic acids and their soaps are similar to the results of Faust and Tallquist[10] who compared the hemolytic action of these acids with that of the oleic acid cholesterin ester which they isolated from the body of the bothriocephalus tape-worm.

[9] *Jour. Exper. Med.*, 1906, viii, 87.

[10] *Arch. f. exper. Path. u. Pharmakol.*, 1907, lvii, 367.

ACTION UPON THE PNEUMOCOCCUS.

For the tests upon the pneumococcus the potassium soaps of the acids were employed. The plan of experiment was the same as that employed for the *in vitro* experiments with sodium oleate described in the first paper.[11] The action of the various soaps was tested alone and in combination with normal goat serum. The results in brief are as follows:

1. There is a close parallel relationship between the lytic activity for red blood cells and for the virulent pneumococci.

2. The difference between the action of these soaps and the action of sodium oleate as previously described is a difference in intensity alone.

3. Potassium linoleate and potassium linolenate are the most active of all soaps which were tested, and they are, respectively, about four and six times as destructive as sodium oleate.

4. Normal goat serum inhibits the action of all of the soaps, but with the same quantity of serum the inhibition is much less in the case of those soaps of the acids having high iodine values; particularly is this true of the potassium soaps of the acids obtained from the phosphorus livers, and of potassium linoleate, and of potassium linolenate.

[11] *Loc. cit.*

CULTIVATION OF TISSUES IN VITRO AND ITS TECHNIQUE.*

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Plates XXXVIII–XLVI.

I. DEFINITION.

A culture consists of a plasmatic medium inoculated with small fragments of living tissues. It is essentially characterized by an active growth of the cells from the original fragment into the plasmatic medium. Epithelial or connective tissue cells wander out in great number from the tissue into the coagulated plasma where they undergo direct or indirect divisions. They cover a wide area of the medium, and are often very densely packed. They grow during a period of time which varies from five or six days to more than twenty days, without any evidence of necrobiosis. The cells which have wandered into or have been born in the plasmatic medium can be transplanted into a fresh medium and produce a new and very luxuriant generation of cells. A culture transplanted into the body of an animal can take and grow rapidly. There is no growth of cells when serum is used instead of plasma as culture medium.

Those characteristics distinguish the culture of tissues from the phenomenon known as the survival of cells. The survival of cells outside of the body has been observed by many investigators; especially by Ljunggren (1), Jolly (2), Carrel (3), Volpino (4), and others. These authors placed pieces of tissues in serum or other fluids and observed the survival of the cells and even some mitotic divisions. But there was no active growth, while at the same time marked necrobiosis took place. Volpino (4) claims to have cultivated sarcoma in horse blood serum. In his experiments there

* Received for publication, January 15, 1911.

was no cultivation of tissues according to our definition; there was only a survival of cells.

II. HISTORY.

The idea of cultivating tissues as previously defined is very far from being new. Many experimenters have already thought of the possibility of growing tissues outside of the body, and several have attempted to develop adequate methods for it. But these attempts were generally not recorded in the literature as they always met with failure. In 1897, Leo Loeb (5) stated that he had cultivated tissue cells outside of the body as well as in the body itself. Although thirteen years have elapsed since his announcement, he has not yet given the results and the technique of his method of cultivation of tissues outside of the body. In 1902, he published his researches on a second method; namely, the cultivation of tissue inside the body. In a series of ingenious experiments, he placed fragments of the skin of embryo guinea pigs in agar and in coagulated serum, and he inserted them into adult guinea pigs. He observed wandering and mitosis of the epithelial cells. In these experiments, the tissues and their media were grafted into a living organism and impregnated with its fluids. They cannot be considered as being strictly equivalent to a culture. Therefore, it is certain that the cultivation of tissues outside of the organism was accomplished for the first time by Harrison, in the Anatomical Department of Johns Hopkins University. In 1907, Harrison (6) demonstrated in a series of splendid experiments that embryonic tissue of the frog, transplanted into coagulable lymph, will develop normally. The central nervous system of a frog embryo, covered with fluid from the lymph sac of an adult frog, produces long nerve fibres. These experiments demonstrated that the nerve fibres are really an outgrowth from a central neurone. But they demonstrated also a very much more important fact, the possibility of growing tissues outside the body. At this time one of us (Carrel) was engaged in the study of the laws of redintegration of the tissues of mammals, and these researches required a method permitting the cultivation, with constant positive results, of mammalian tissues outside the body. Therefore he resolved to use

for this purpose the method of Harrison, after it had been adapted to the culture of mammalian tissues. Then, at Yale University, and under the guidance of Professor Harrison himself, Burrows (7) began to acquire and improve the technique of tissue cultivation by using plasma from the blood instead of lymph. Afterwards, he succeeded in adapting the method to the cultivation of tissues of the chick embryo. After having established this very important modification, he cultivated outside of the body the central nervous system, the heart and the mesenchymatous tissue of the chick embryo, or in other words, of a warm-blooded animal. Then in September, 1910, at the Rockefeller Institute, we succeeded in cultivating *in vitro* adult tissues of mammals. The technique was rapidly improved as to details, and the results became practically uniformly positive. We used, at first, the culture method of Harrison, that is, of small pieces of tissue suspended in a hanging drop of plasma. Afterwards, we developed a method of culture on a plate, which permitted us to grow large quantities of tissues. In the intervening few months, it became, therefore, possible to observe many new facts. It was found at first, that almost all the adult and embryonic tissues of dog, cat, chicken, rat, and guinea pigs could be easily cultivated *in vitro* (8). According to their nature, these tissues generate connective or epithelial cells, which grow into the plasmatic medium in continuous layers, or in radiating chains (plate XXXVIII, Fig. 1). The tissue fragments may surround themselves completely with dense new tissue, or, on the contrary the new cells may spread over the surface of the medium. We observed the direct division of the nuclei during the life of the cells, and many karyokinetic figures in the fixed and stained cultures (plate XXXIX, Fig. 2). Other experiments showed that the life *in vitro* of the tissues, which varies from five days to about twenty days, can be prolonged by secondary and tertiary cultures (9), and that a second generation of thyroid, splenic, and sarcomatous cells can be obtained from cells which have developed outside the body (10). We succeeded quickly also in cultivating malignant tissues such as the Rous (11) chicken sarcoma (Fig. 1), the Ehrlich (plate XL, Fig. 4; plate XLI, Fig. 5; plate XLII, Fig. 6) and Jensen sarcoma of the rat, a primary carcinoma of the breast

(dog), and two human tumors, a sarcoma of the fibula (12), and a carcinoma of the breast. A culture *in vitro* of the Rous sarcoma transplanted into a chicken, caused the development of a sarcoma. Meanwhile the method has been applied successfully in the Laboratory of Professor MacCallum by Drs. Lambert and Hanes (13), who cultivated the Ehrlich sarcoma of the rat. In December, 1910, and in January, 1911, we applied the method of cultivation of tissues *in vitro* to several problems of the redintegration of normal tissues and of the biology of malignant tumor (14). Dr. Ruth found that fragments of skin with a small open wound in the center undergo *in vitro* a process resembling normal cicatrization. This new method of observing cicatrization of tissues outside of the body is very valuable for the study of the redintegration of normal tissues.

Dr. Jolly (15) of the Collège de France, in a recent communication to the Society of Biology of Paris, announced that we had not succeeded at all in cultivating tissues *in vitro,* and that we had observed only necrobiosis of the tissues and survival of a few cells.

III. TECHNIQUE.

The growth of tissue cells is obtained when small fragments of living tissue are placed at the proper temperature in fluid plasma, which will coagulate immediately. The cultures belong to two types: the small cultures in a hanging drop, similar to those of Harrison, and the large cultures in the surface of a plate, which can be compared to the plate cultures of bacteria. Theoretically, the technique is very simple, and it is very easy to obtain some growth *in vitro* of the tissues. But in order to obtain results which are uniformly positive, and which can be used for comparisons, the technique must be more elaborate in its details.

Tissues, especially the higher adult mammalian tissues, are easily killed by drying, chilling, and rough handling during the preparation of the culture. Bacterial infection is also detrimental to tissue growth. A rigid asepsis is necessary for the preparation of any tissue culture. The culture must be made in a warm humid operating room with the same care and rapidity as a delicate surgical operation. If the method is to give uniform results, not only must

the above precautions be closely followed but also the perfect teamwork of well-trained assistants is necessary.

In the following we shall describe the preparation of plasma, of the tissues, of the culture, and the methods of observing the growth of the cells.

1. *Preparation of the Plasma.*—The plasma is prepared from the blood of the animal whose tissues are to be cultivated or from another animal from the same species. Pure plasma or oxalated plasma can be used. Pure plasma gives far better results, and is to be preferred to oxalated plasma.

Pure plasma is prepared by a method similar to that used by Delezenne and by Gengou. The blood is taken from an artery or from a vein. When dogs, cats, chickens, guinea pigs, and rats are used, the carotid artery is ordinarily selected. For human beings, the blood is easily obtained from one of the superficial veins of the arm. The animal is etherized and the vessel is exposed and dissected from the surrounding tissue. The wall of the blood vessel is rubbed with dry gauze, and covered with olive oil, the circulation is then interrupted by a *serre fine,* the vessel wall is opened laterally, and a glass cannula, previously sterilized in olive oil is inserted into the lumen of the vessel. It is also possible to use on human beings a needle sterilized in olive oil and inserted through the skin into the vein.

The blood is collected in small tubes, carefully coated with paraffine, which have been previously cooled at 0° C. The tubes are immediately corked, placed in large tubes filled with ice, centrifugalized for five minutes and deposited in a small ice-box at 0° C. The supernatant plasma is removed with pipettes coated with paraffine. It is generally used immediately, but it can be preserved for some time in a fluid condition if it is kept at a low temperature. Chicken plasma can be preserved for more than one week, human and dog plasma for a few days, while rat plasma always coagulates after a few hours.

Oxalated plasma was also used by Burrows (7) in his earlier work on the chick embryo. Sufficient blood was added to a 1 per cent. solution of sodium oxalate, making the solution 0.1 per cent. At the time of use the sodium oxalate was precipitated quantitatively

from this plasma by the addition of calcium chloride, after which coagulation occurred. Although oxalated plasma does not give as good results as pure plasma, it can be used in cases of necessity.

2. *Preparation of the Tissues.*—The tissues used for cultures must be in normal condition. They are best if taken directly from the living animal or from an animal immediately after death. Positive results can still be obtained, however, when the tissues have been deprived of circulation for more than thirty minutes, but it is always better to put the tissues in the plasma as soon as the circulation is interrupted. With a cataract knife and a fine needle, a small fragment of tissue is dissected from the animal and placed on a glass plate. This piece of tissue is rapidly cut into small pieces about the size of a millet seed and transferred on the point of a needle to the surface of a cover glass. For the large cultures, the tissue is cut into small pieces with sharp scissors, or what is still better, into thin, broader pieces with a razor.

It must be remembered that Cristiani has demonstrated that a small piece of thyroid dies if exposed to the drying action of the air for more than ten seconds. Therefore, the section and the handling of the tissues must be very rapid, otherwise the tissue is killed. The dissection of the tissue may be made in a drop of serum, in order to prevent that accident.

3. *Preparation of the Culture.*—Two types of cultures have been prepared, the small hanging drop culture and the large plate culture. The small cultures are similar to those used by Harrison (6). One or two small pieces of tissue are transferred to a cover glass and quickly covered with a drop of plasma. It is best to spread the plasma in a thin layer over the cover glass. This is done with the needle before coagulation occurs. The cells grow, then, in a few planes (plate XLIII, Fig. 7) and in areas about the tissue. If the drop is thick the cells grow in many planes and it is difficult to measure the area of growth or to photograph and observe the growing cells. The cover glass is then inverted over a hollow slide of sufficient depth to prevent the drop from touching the bottom, and sealed to the slide with paraffin to prevent drying. The finished slide is immediately placed in a small electric incubator which is used for transferring the cultures from the operating room to

the large incubator in the room where the study of the cultures are made. Coagulation of the plasma takes place either immediately upon the addition of the tissue or soon after the slides are placed in the warm oven.

To grow tissues on a large scale, the same general technique is used. A rigid asepsis here is most necessary as it is very easy to infect these large cultures. An entire chicken fetus of fifteen days, or small mammalian fetuses cut into small fragments may be used for these cultures. These fragments are spread in a thin layer over the surface of a large black glass plate and covered quickly with fluid plasma. As soon as coagulation of the plasma has taken place, the plates are placed in glass boxes with cotton sponges soaked in water, which preserve the proper humidity (plate XLVI, Fig. 11). The boxes are then carefully sealed with paraffine and kept in such a position that the fluid products of the culture may drain to the bottom.

4. *Preservation and Observation of the Cultures.*—During their growth, the cultures can be removed from the incubator for a few seconds without danger to their life. Certain tissues, like malignant tumor or spleen (Fig. 1 and plate XLIII, Fig. 7), grow and extend so widely that their condition can be observed without the use of the microscope. On a hollow slide, the new tissue of a culture of spleen appears as an opalescent area surrounding the primitive fragment. Even the beginning of growth can be diagnosed by the appearance on the sharp edges of the fragment of a very faint and narrow gray band. In the culture on plates the appearance of a whitish color around the fragments of the tissues shows that they are growing. But it is safer to make a few control cultures in hollow slides and to observe their growth with the microscope.

For the study of the cultures, we use a microscope placed in a warm stage, the temperature of which is kept constant. The slides can be kept under the microscope for a long time, if necessary, without any danger to the life of the tissue. Before the beginning of the growth, the fragment of tissue appears as an opaque, sharply outlined mass in the clear medium. In the surrounding clear medium, the growing cells are easily detected. They appear as fusi-

form or polygonal bodies isolated or united by filaments or densely packed together (Fig. 7 and plate XLIV, Fig. 8). Generally the cytoplasm is filled with refractile granules. The nucleus stands out as a clear and homogenous area (plate XXXIX, Fig. 3). It contains one or more darker nucleoli. When the cells grow in continuous layers, for instance in cultures of thyroid gland (plate XLV, Fig. 10), their individual outlines cannot be observed. They appear as a layer of small granulations, surrounding a great many clear spots. When the culture is fixed and stained by hematoxylin, the outlines of the cells become distinct, and the clear spots appear to be the nuclei of the cells (Fig. 3). Often the movement of the living cells, their modification in shape, and the division of their nucleus can be readily and directly observed. Nuclear budding with formation of multinuclear cells have frequently been observed in the spleen (plate XLIV, Fig. 9).

Camera lucida drawings of the cells can be made when the tissues develop slowly like cartilage or peritoneum. But even in these cases, the motion of the cells and the changes in their shape require that the sketches be made rapidly. The growth of sarcoma or of spleen is often so rapid that it renders impossible an accurate camera lucida drawing.

The best method of recording the morphology of the living cultures is to photograph them. But this is often very difficult, because the new tissue is dense or the cells are faintly seen; and chiefly because the cells do not grow on the same plane. Generally in a very actively growing culture no cell can be seen distinctly. They are disposed in chains closely packed and radiating from the original fragment as a center (Fig. 1). Even when the outlines of the cells can be distinguished easily under the microscope, a sharp photograph of them may be impossible if they are surrounded by cells which have grown on slightly different planes. Mitotic figures have never been detected in a living culture, and they have become visible only after staining the fixed specimen (Fig. 2).

For exact cytologic study, the cultures are fixed and stained. The cover glass, to which the culture is adherent, is separated from the hollow slides and immersed in corrosive sublimate, acetic acid, or formalin, or the various preparations of potassium bichromate

solutions. Afterwards, they are stained in hematoxylin. When the culture medium is spread on the cover glass in a very thin layer, and when the culture is not too old, the cells appear very distinctly and all their structural details are easily observed (Figs. 5, 6, 8, 9, 10). In many places, beautiful mitotic figures are present (Fig. 2). When the plasmatic medium is thick, and when the cells have grown in many different planes, serial sections of the hardened culture are required.

The histological characteristics of the large cultures on glass plates can be studied only by serial sections. The purpose of a culture on a plate is not the observation of the morphology of the cells, but the study of the dynamic changes undergone by the cells during their life outside of the organism, and the nature of their secretions.

When the technique is applied carefully in all its details, the results of the cultures are practically uniformly positive. If some of the details are neglected, the tissues do not grow or their growth is altered. Great accuracy in the technique is required when the method of cultivation of tissues *in vitro* is employed for the study of such important problems as the redintegration and growth of normal tissues and the growth of malignant tumors.

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EXPLANATION OF PLATES.

Plate XXXVIII.

Fig. 1. Living culture of the Rous chicken sarcoma, twenty-four hours old. The central opaque mass represents the original fragment of tissue. The new cells are radiating in great numbers from the tissue. The irregular outer dark areas are reflections from water of condensation on the bottom of the culture.

Plate XXXIX.

Fig. 2. Culture of Wolfian body of a chick embryo. Mitosis of the new-grown cells. Stain hematoxylin.

Fig. 3. Isolated living connective tissue cells. The cytoplasm of these cells is filled with refractile fat granules. Nucleus is the clear oval area. In some of the nuclei faintly staining nucleoli can be made out.

Plate XL.

Fig. 4. Culture of the Ehrlich rat sarcoma. The central and completely opaque mass is the original tumor fragment. The new cells are arranged irregularly throughout the surrounding medium. Stain hematoxylin.

Plate XLI.

Fig. 5. Small area of the new-grown cells of the living culture of the Ehrlich rat sarcoma shown in Fig. 4.

Plate XLII.

Fig. 6. Photograph of the culture of the same cells as seen in Fig. 5. Stain hematoxylin.

Plate XLIII.

Fig. 7. Culture of spleen.

Plate XLIV.

Fig. 8. Same culture as Fig. 7. An area of the new-grown connective tissue cells. Stain hematoxylin.

Fig. 9. Isolated cells from a culture (Fig. 7) of adult spleen (chicken). The cells are chiefly multinuclei and filled tightly with large fat granules. Stain hematoxylin.

Plate XLV.

Fig. 10. Culture of thyroid (adult dog). A layer of epithelial cells spreading out from the border alveoli of the thyroid. Isolated connective tissue cells are seen in the clear medium beyond. Stain hematoxylin.

Plate XLVI.

Fig. 11. Large plate culture in its sealed moist chamber.

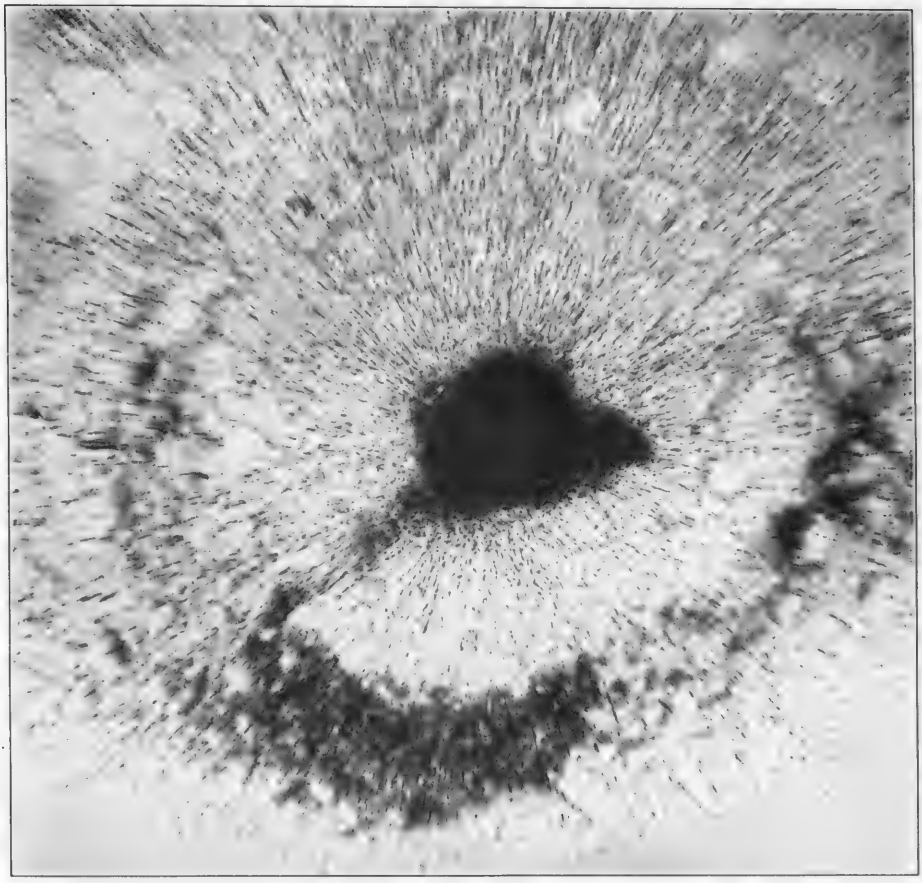

FIG. 1.

FIG. 2.

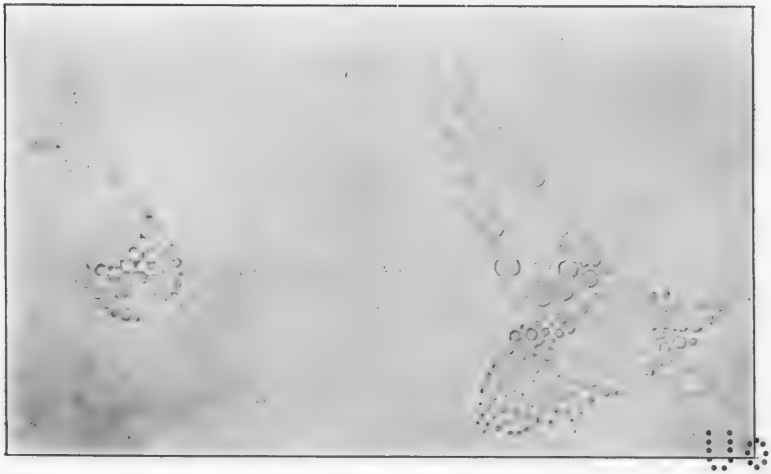

FIG. 3.

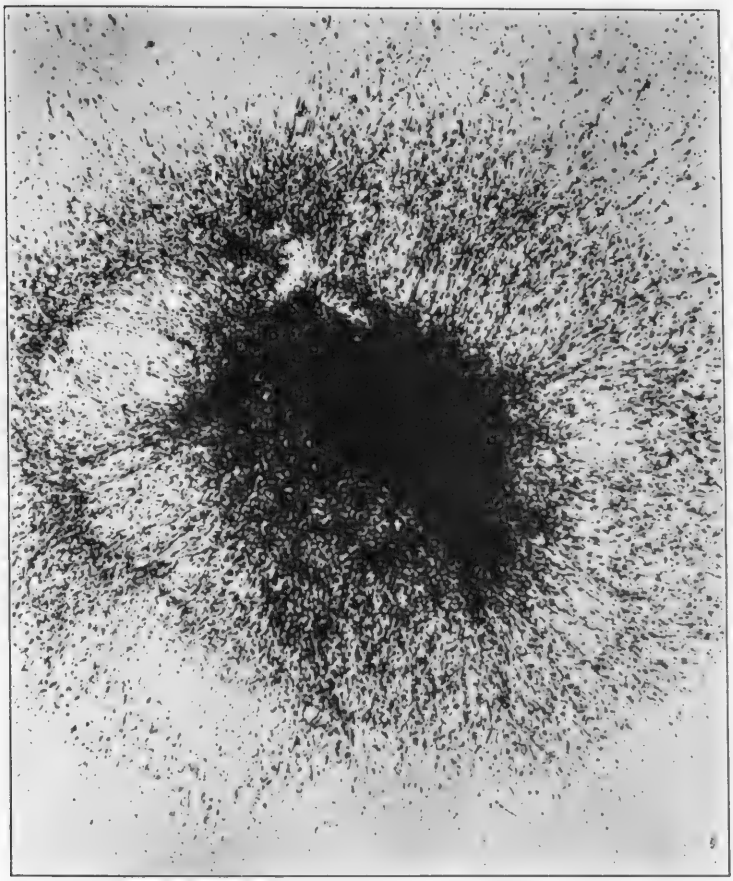

FIG. 4.

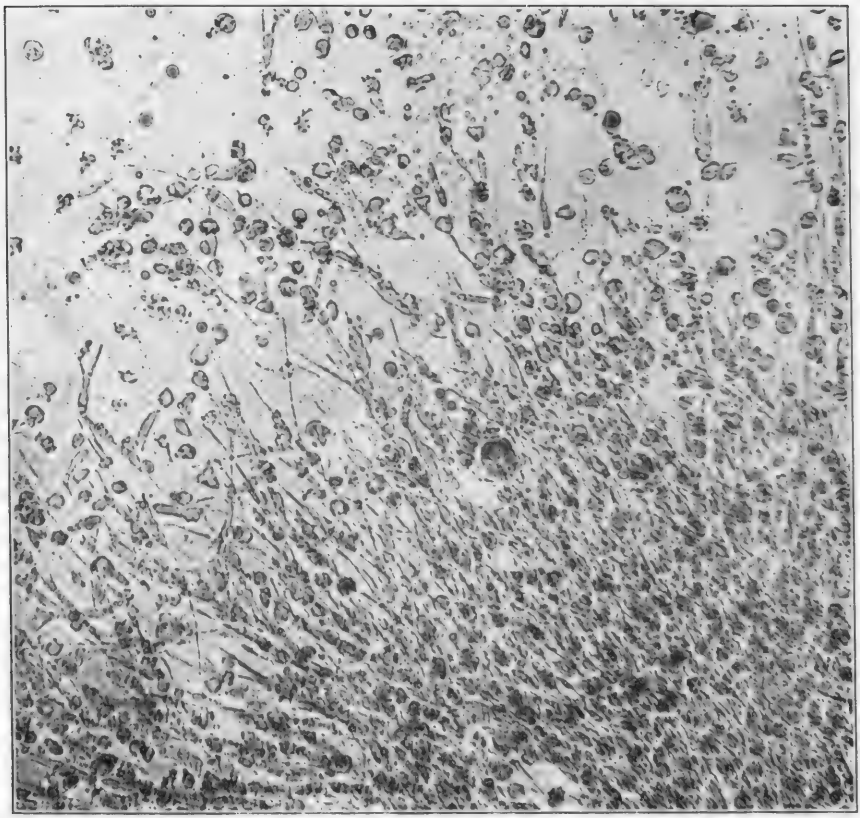

FIG. 5.

FIG. 6.

FIG. 7.

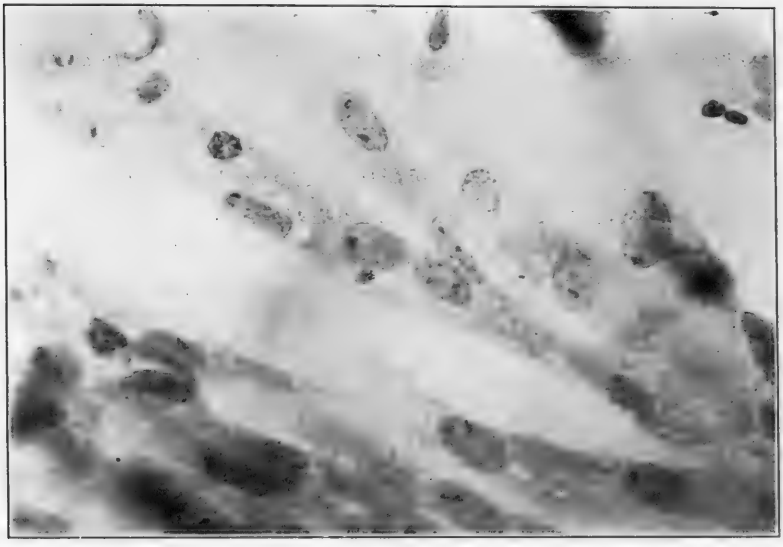

FIG. 8.

FIG. 9.

U of M

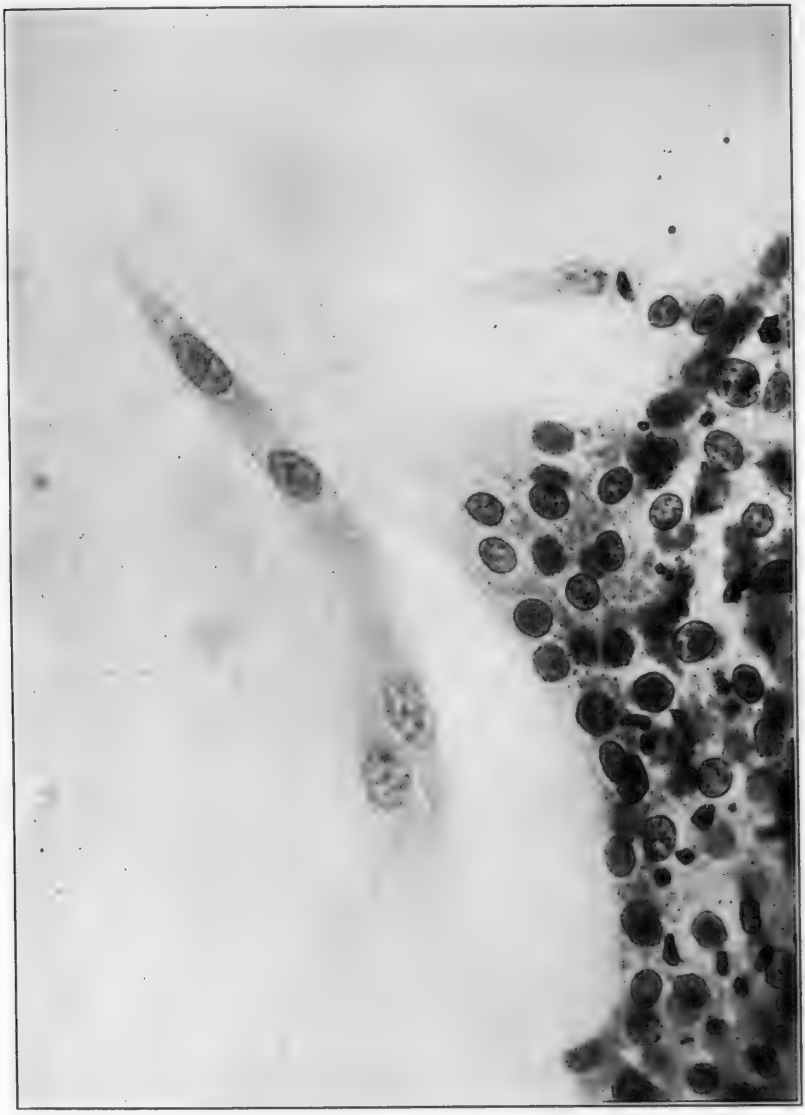

FIG. 10.

U of M

UoPM

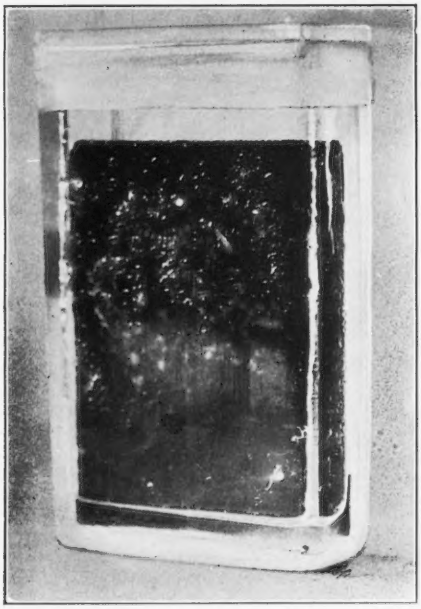

FIG. 11.

U of M